AD_____

GRANT NUMBER DAMD17-95-1-5015

TITLE: Estrogen Receptor Mutants/Variants in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Leigh C. Murphy, Ph.D.

CONTRACTING ORGANIZATION: University of Manitoba
Winnipeg, Manitoba, Canada R3E-OW3

REPORT DATE: December 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1998	Final (15 Nov 94 - 14 Nov 98)

4. TITLE AND SUBTITLE
Estrogen Receptor Mutants/Variants in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-95-1-5015

6. AUTHOR(S)
Leigh C. Murphy, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Manitoba
Winnipeg, Manitoba, Canada R3E-OW3

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Our results suggest that estrogen receptor mediated signal transduction is complex and multifaceted in human breast cancer. We have identified the frequent expression of several alternatively spliced variants of the classical ER-α as well as the expression of a second novel ER gene (ER-β) and several (ER-β) variants, at least, at the mRNA level, in both normal and neoplastic human breast tissues. Further we have both published and preliminary data showing alterations in the relative expression of several of these ER-like molecules during human breast tumorigenesis and breast cancer progression. In particular marked alterations of the relative expression of several ER-α variant mRNAs and the relative expression of ER-α and (ER-β) mRNA occurs between normal breast tissue and primary breast tumors. Our data suggest that marked alterations of several players involved in estrogen signal transduction occur during breast tumorigenesis in particular, and these changes may contribute to the altered estrogen sensitivity that appears to occur during breast tumorigenesis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Estrogen Receptor, Molecular Pathology, Mutations/Variants, Tamoxifen Resistance, Endocrine Resistance, Molecular Biology, Humans, Breast Cancer	196
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

__✓__ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__✓__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

__✓__ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature

10 Dec 1998
Date

4. TABLE OF CONTENTS.

5. INTRODUCTION:

The goal of this research is to address the role of variant/abnormal estrogen receptor (ER) expression in the progression of human breast cancers from hormone dependence to independence. The progression of breast cancer from hormone dependence to independence is a clinically significant problem since it limits the effectiveness of the relatively non-toxic hormonal therapies such as antiestrogens and progestins (1). The hormone-dependent phenotype is characterized by the presence of ER in the breast tumor, but only 50% of receptor positive breast tumors respond to endocrine therapies and of those which initially respond a significant proportion will eventually develop resistance to these therapies. Furthermore, the development of resistance to endocrine therapy occurs despite the continued expression of ER in the tumor, in at least 50% of cases. It is the molecular mechanisms of this form of resistance i.e. the steroid receptor positive/hormone resistant human breast tumors, that this research proposal addresses. Elucidation of these mechanisms will provide information necessary either to prevent the occurrence of hormone resistance, reverse it or develop new treatments for the resistant tumors. As well, novel treatment response markers in human breast tumors are likely to be identified. Although multiple mechanisms are likely to be involved in hormone resistance and progression to hormone independence (1) in human breast cancer, this grant proposal focuses on one possible mechanism: the involvement of variant and/or abnormal forms of ER.

The hypothesis to be tested is that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

The specific aims to address this hypothesis are:

1. To systematically investigate alterations that occur in the ER mRNA in human breast cancers.
2. To characterize structurally and functionally those abnormal ER mRNAs occurring most frequently and determine their involvement in the development of hormone independence and progression in HBC.
3. To develop specific tools to investigate the expression of the corresponding proteins that may be translated from altered or variant ER mRNAs.
4. To assess the biological significance of ER variants in human breast cancer by determining the relationship between the level of expression of ER variants in human breast cancer biopsies and the expression of the normal ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors.

6. BODY:

Task 1. To systematically investigate alterations that occur in the ER mRNA in human breast cancers. We have systematically investigated alterations that occur in the estrogen receptor (ER) mRNA in human breast tumors. Several manuscripts and abstracts reporting these studies have been published (see references 2-5). Another estrogen receptor gene, estrogen receptor-β (ER-β) was reported during the tenure of this current grant (6). The ER-β protein has similarities to the classical ER referred to as ER-α, in terms of structure and function. Both these proteins have a high degree of conservation of the DNA and ligand binding domains, while the A/B, hinge and F domains are not conserved. The tissue specific expression of ER-α and ER-β although not identical appears to overlap in some cases (6). The expression of ER-β in normal or neoplastic mammary tissue was not reported however, and it became extremely important to determine if ER-β, an ER isoform/variant, is expressed in breast cancers and therefore could potentially contribute to estrogen signal transduction in this tissue. We investigated this possibility and were the first to publish the detection of ER-β mRNA in several human breast tumor biospy samples and several human breast epithelial cell lines using reverse transcription polymerase chain reaction (RT-PCR) analysis (7). The expression of ER-β was not correlated with that of ER-α and both ER positive and negative cell lines as determined by ligand binding assay, expressed ER-β mRNA. However, some breast tumors and some cell lines coexpress ER-β and ER-α mRNA. Our data support a role for ER-β in human breast cancer. Further, we have now reported that the presence of ER-β mRNA in normal human breast tissue (8) and have also demonstrated the presence of variant forms of ER-β mRNA in several human and mouse tissues (8), although species specific differences were found to occur.

We have developed a multiplex RT-PCR assay, in order to compared the relative expression of ER–α and ER–β mRNA between adjacent samples of normal breast tissue and their matched primary breast tumors obtained from 18 different patients. Within this cohort, 7 tumors were ER negative and 11 tumors were ER positive, as determined by ligand binding assay. No differences in the ratio of ER-α/ER–β expression were observed in the ER negative cohort. However, in the ER positive cohort, a significantly ($p<0.02$) higher ratio ER–α/ER–β was observed in the tumor compared to the normal tissue component. Our data revealed that the increase in ER–α/ER–β ratio was primarily due to a significant ($p<0.05$) increase in ER–α mRNA expression in conjunction with a lower ER–β mRNA expression in the tumor compared to the normal compartment in some but not all ER positive cases. These results suggest that the role of ER–α and ER–β driven pathways and/or their interaction change during breast tumorigenesis (9).

We have also demonstrated that previously identified exon 8 deleted ER–β variants, called ER–β 2 and ER–β 5 (10), are expressed in both normal and neoplastic human breast tissues (11).

Task 2. To develop quantitative RT-PCR assays for measuring selected ER mRNA variants in RNA isolated from microdissected regions of human breast tumors. Several different assays were developed, each with different advantages and limitations. These results have been previously published (4, 12-16). The type of assay used, was dictated by the question asked and practical issues. These assays are being used to assess the biological significance of ER-α variants in human breast cancer under Task 5. However, in order to compare some of our current data with that obtained previously (17), it was necessary to validate the comparsion of our RT-PCR based assays with RNase protection assays. The results of this validation are in press (18). Due to our demonstration of the expression of ER-β mRNA in human breast tumors and the possible differential regulation of ER-β and ER-α we have developed a multiplex assay to determine the relative expression of these two ER genes in human breast tissues (9). As well we have developed and validated an RT- triple primer PCR assay to simultaneously determine in small samples of breast tissue the relative expression of the wild type ER–β and the two variant ER–β mRNAs deleted in exon 8 which we have found highly expressed in human breast tissues (11).

Task 3. To characterize structurally and functionally those variant ER mRNAs occurring most frequently in human breast cancer. Eukaryotic expression vectors for several of the variant and mutant ER-α mRNAs have been constructed and used to determine the ability of the predicted protein(s) to bind ligand, have transcriptional activity alone or alter transcriptional activity of the wild type ER-α (19). Previously we had developed a model of estrogen independence in human breast cancer cells (20). Two molecular alterations were identified in this model: 1. There is a marked up-regulation of the relative expression of an exon 3+4 deleted ER-α mRNA in the estrogen independent cells (T5-PRF) compared to the T5, estrogen responsive parent cells (19). The increased relative expression of this ER-α variant mRNA correlated with a significant increase in the basal, estrogen independent activity of the wild type ER-α, as determined by transient transfection of an ERE-tk-CAT reporter gene and the measurement of endogenous progesterone receptor (PR) expression (19), in the T5-PRF cells. An expression vector containing the exon 3+4 deleted ER-α cDNA was constructed. The *in vitro* transcribed/translated protein does not bind estradiol and does not interact with an ERE in gel shift assays. But, when expressed following transient transfection in the estrogen responsive parent T5 cells, a dose-dependent increase in basal, ligand independent activity of the endogenous wild type ER-α occurred (19). However, when the exon 3+4 deleted variant ER-α expression vector is transfected alone into MDAMB231 (ER-

α negative human breast cancer) or MCF10A1 (ER-α negative nontumorigenic, immortalized human breast epithelial) cells with an ERE-tk-CAT reporter the variant protein has no detectable transcriptional activity (19). Co-transfection of a constant amount of wild type ER-α expression vector and increasing exon 3+4 deletion ER-α expression vector, increased the ligand independent ER-α induced ERE-tk-CAT activity in both MCF10A1 and MDAMB231 (19). Although we can detect recombinantly expressed variant ER-α protein following transfection into ER- cells we have been unable to unequivocally determine any increased relative expression of a variant ER-α protein consistent with that predicted to be encoded by an exon 3+4 deleted ER-α variant mRNA in T5-PRF cells compared to T5 cells. The exon 3+4 deleted ER-α protein is predicted to encode an approx. 49 kDa protein and although an immunoreactive ER-α protein can be detected at around 50 kDa in both T5 and T5-PRF cells it does not directly correlate with exon 3+4 deleted ER-α variant mRNA level. Furthermore, other predicted variant ER-α proteins (21) would also be around 50 kDa, as is a commonly occurring proteolytic fragment of the wild type ER-α protein. 2. A second molecular alteration correlated with the estrogen independent phenotype of the T5-PRF cells has also been identified (22). We have determined that T5-PRF cells have an increased expression of activated mitogen activated protein kinase (MAPK) compared to the estrogen responsive T5 parent line (22). Since MAPK has been shown to phosphorylate and activate the wild type ER-α protein in a ligand independent fashion (23) it is possible that it's increased activity has a role together with the increased expression of a variant ER-α protein in the estrogen independent phenotype of T5-PRF cells.

We have over-expressed a truncated ER-α protein (clone 4 truncated ER-α protein) in estrogen responsive MCF-7 cells. Our preliminary data indicate that this does not effect estrogen sensitivity of these cells as determined by exponential growth of the cells or PR expression or activation of ER-α induced transcription of an ERE-tk-CAT reporter plasmid. This contrasts with our previous observation that increased relative expression of the clone 4 truncated ER-α variant mRNA was significantly correlated with poor prognostic characteristics in human breast tumors and lack of sensitivity to endocrine therapies as defined by lack of PR expression (17). These data suggest that overexpression of a single ER-α variant protein alone is insufficient to confer estrogen independence and antiestrogen resistance to breast cancer cells. However, these data would be consistent with the need for multiple alterations being involved in altered estrogen and antiestrogen sensitivity as seen in the T5-PRF cell line (19,22).

Task 4. <u>To develop specific tools (antibodies) to investigate the expression of the corresponding proteins that may be translated from variant ER-α mRNAs.</u>

A synthetic peptide containing the novel 6 amino acids present in the predicted clone 4 ER-α protein was conjugated to KLH (Chiron Inc) and this antigen was used to raise antibodies to the clone 4 ER-α variant protein in rabbits (National Biological Labs). The antisera have been prescreened against the synthetic peptide, and the most promising antisera were used to screen Cos-1 and MCF-7 cells stably transfected with the clone 4 variant ER-α transgene. Our preliminary data show that the antisera can detect a 24 kDa protein present in transgene transfected Cos-1 and MCF 7 but not in vector alone transfected cells. Further, a similar sized protein is also detected with H226 antibodies (which detects an N-terminal epitope of the wild type ER-α which is predicted to be present in the clone 4 variant ER-α protein). These data suggest that the antisera contains specific antibodies to the clone 4 ER-α protein. Various dilutions of the antiserum appear to detect other proteins on Western blots. Immunoglobulin isolation and small batch affinity purification was done and the resulting antibody has markedly reduced background/non-specific activity. This procedure is being scaled up to generate sufficient affinity purified antibody for Western blot analysis of breast tumor extracts and to determine if this antibody is useful for immunohistochemical analyses and immunopreciptation. Following the satisfactory completion of this characterization we will analysis human breast tumor samples to determine the presence of clone 4 ER-α-like epitopes and proteins *in vivo*.

Previously, we have provided indirect evidence to support the detection of C-terminally truncated ER-α variant proteins which could correspond to those predicted to be encoded by ER-α variant mRNAs. This evidence was based on the correlation of increased expression of ER-α variant mRNAs encoding C-terminally truncated proteins with increased detection of C-terminally truncated ER-α like proteins in breast tumors. C-terminally truncated ER-α like proteins were determined by the increased immunohistochemical (IHC) signal using an antibody which recognizes an N-terminal epitope in the wild-type ER-α protein (expected to be present in many ER-α variants), compared to the IHC signal using an antibody which recognizes a C-terminal epitope in the wild type ER-α protein (not expected to be present in many ER-α variants) (24,25). In addition we have shown experimentally that we can cause discrepancies in the IHC determination of ER status due to increased expression of C-terminally truncated ER-α variant proteins using transient transfection of expression vectors (26). The data suggest that the ER-α variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER-α measured by immunodetection methodologies. Further, the data are consistent with the ability of some ER variant mRNAs to be stably translated *in vivo*. The data do not unequivocally prove that ER-α variant proteins corresponding to known ER-α variant mRNAs can be detected *in vivo*.

Task 5. To assess the biological significance of ER variants in human breast cancer. Available studies provide evidence for an extensive and complex pattern of alternative splicing associated with the ER-α gene, which seems to be altered during breast tumorigenesis (4,12,14,27). Further our data suggest that the mechanisms generating alternatively spliced forms of ER-α are unlikely to be due to a generalized deregulation and/or alteration of splicing processes within breast tumors (28).

We have previously shown that alteration of expression of some variant ER-α mRNAs was found to occur during breast tumorigenesis (12,14). These studies were done using unmatched samples of breast tumors and normal breast tissues. We have now compared the relative expression of an exon 3 deleted, an exon 5 deleted and the truncated clone 4 ER-α variant mRNAs in primary breast tumors and their matched adjacent normal breast tissues (29). Our data confirm the previous observations that the relative expression of the exon 5 deleted and the clone 4 truncated ER-α variant mRNAs is increase in breast tumor subsets compared to their matched normal tissues (29). However, this study also demonstrated that the exon 3 deleted ER-α variant mRNA was significantly decreased in breast tumors compared to their matched normal tissues. The data suggest that multiple alterations of the relative expression of ER-α variants occurs during breast tumorigenesis and may contribute to altered estrogen responsiveness during breast tumorigenesis.

We have also previously documented altered ER-α variant mRNA expression during breast cancer progression (4, 17). In particular we showed that the expression of the truncated, clone 4 variant ER-α mRNA relative to wild type ER-α mRNA was significantly increased in a group of primary breast tumors with poor prognostic features compared to a group of primary breast tumors with good prognostic features (17). "Poor" prognostic features included the presence of lymph node metastases at the time of surgery, large tumor size, lack of PR expression and high proliferative index, while "good" prognostic features were lack of nodal involvement, small tumor size, PR positivity and low proliferative index. This suggested that altered ER-α variant expression may be a marker of a more aggressive phenotype. We investigated this possibility further by comparing the over-all pattern of deleted ER-α variant expression between matched primary tumors and their concurrent lymph node metastases, as well we compared the relative level of expression of several individual ER-α variant mRNAs in these matched tumor samples. Our data (18) suggest that both the pattern of ER-α variant expression and the relative level of expression of three individual ER-α variants are conserved in primary breast tumors and their matched, concurrent lymph node metastases. These findings are not inconsistent with our previously published data in which the relative expression of at least one ER-α variant was significiantly increased in primary tumors with poor prognostic characteristics, which included having concurrent lymph node metastases,

as compared to primary tumors without concurrent lymph node metastases (17). The primary tumors in our current study by definition fall into the previously described poor prognostic group since they are all primary breast tumors with concurrent lymph node metastases.

To assess the potential biological significance of ER-β expression in human breast cancer we have measured the relative expression of ER–α and ER–β mRNA between adjacent samples of normal breast tissue and their matched primary breast tumors. A significantly ($p<0.02$) higher ratio ER–α/ER–β was observed in the tumor compared to the normal tissue component of those cases where the tumor was ER+ as defined by ligand binding analysis. Our data revealed that the increase in ER–α/ER–β ratio was primarily due to a significant ($p<0.05$) increase in ER–α mRNA expression in conjunction with a lower ER–β mRNA expression in the tumor compared to the normal compartment in some but not all ER positive cases. These results suggest that the role of ER–α and ER–β driven pathways and/or their interaction change during breast tumorigenesis (9). Despite the observation that ER–β mRNA is downregulated in many human breast tumors, the level of ER-β mRNA appeared to vary amongst breast tumors (7). These observations raised the question of whether the expression of ER-β in breast tumors was correlated with known prognostic and endocrine treatment response markers. Therefore the relationship of ER-β mRNA expression to ER and PR status (as determined by ligand binding analysis) was investigated. The level of ER-β mRNA was found to be significantly lower in PR+ tumors compared to PR - tumors ($p=0.036$) and no association with ER status was found. Subgroup analysis showed that ER-β mRNA expression in ER+/PR+ breast tumors was significantly less than in ER+/PR- ($p=0.009$), ER-/PR+ ($p=0.029$) and ER-/PR- ($p=0.023$) groups. Interestingly, the ER-β mRNA expression was specifically decreased by progestin in T-47D breast cancer cells. The inverse relationship between PR (a good prognostic variable and a marker of response to endocrine therapies) and ER-β suggests that although ER-β is often downregulated in human breast tumors compared to normal human breast tissue (9), its maintainance and/or increased expression in some breast tumors may correlate with a poorer prognosis and the likelihood of failure of response to endocrine therapies such as antiestrogens. This remains to be tested in samples of breast tumors from patients known to have responded or not to have responded to endocrine therapies, in clinical trials. The data suggest the possibility that expression of ER-β in human breast tumors is a marker of endocrine therapy responsiveness (30).

7. CONCLUSIONS.

Our results suggest that estrogen receptor mediated signal transduction is complex and multifaceted in human breast cancer (21). We have identified the frequent expression of several alternatively spliced variants of the classical ER-α as well as the expression of a second novel ER gene (ER-β) and several ER-β variants, at least, at the mRNA level, in both normal and neoplastic human breast tissues. Further we have both published and preliminary data showing alterations in the relative expression of several of these ER-like molecules during human breast tumorigenesis and breast cancer progression. In particular marked alterations of the relative expression of several ER-α variant mRNAs and the relative expression of ER-α and ER-β mRNA occurs between normal breast tissue and primary breast

tumors. Our data suggest that marked alterations of several players involved in estrogen signal transduction occur during breast tumorigenesis in particular, and these changes may contribute to the altered estrogen sensitivity that appears to occur during breast tumorigenesis.

8. REFERENCES:

1. Katzenellenbogen BS. Antiestrogen resistance: mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. J Natl Cancer Inst 83: 1434-1435, 1991.

2. Murphy LC, Wang M, Coutts A, Dotzlaw H. Novel Mutations in the Estrogen Receptor mRNA in Human Breast Cancers. J Clin Endocrinol Metab 81: 1420-1427, 1996.

3. Wang M, Dotzlaw H, Fuqua SAW, Murphy LC. A point mutation in the human estrogen receptor gene is associated with the expression of an abnormal estrogen Receptor mRNA containing a 69 novel nucleotide insertion. Breast Cancer Res Treat 44: 145-151, 1997.

4. Leygue ET, Huang A, Murphy LC, Watson PH . Prevalence of estrogen receptor variant messenger RNAs in human breast cancer. Cancer Res 56: 4324-4327, 1996.

5. Douglas D, Murphy LC. Functional Characterization of Novel Inserted Estrogen Receptor (ER). Abstract P1-522, 79th Annual Meeting of the Endocrine Society. Minneapolis, June 1997.

6. Mosselman S, Polman J, Dijkema R. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Letters 392: 49-53, 1996.

Kuiper G, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. U.S.A. 93: 5925-5930, 1996.

7. Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor-beta in human breast tumors. J Clin Endocrinol Metab 82: 2371-2374, 1997.

8. Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC, Watson PH. Estrogen receptor-beta mRNA variants in human and murine tissues. Mol Cell Endocrinol 138: 199-203, 1998. Reprint attached, **Appendix 1.**

9. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Altered estrogen receptor alpha and beta mRNA expression during human breast tumorigenesis. Cancer Res 58: 3197-3201, 1998. Reprint attached, **Appendix 2.**

10. Moore JT *et al.,* Cloning and characterization of human estrogen receptor-beta isoforms. Biochem Biophys Res Commun 247: 75-78, 1998.

11. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Expression of estrogen receptor beta-1, beta2 and beta-5 mRNA in human breast tissue. Manuscript attached, **Appendix 3.**

12. Leygue E, Watson PH, Murphy LC. Estrogen receptor variants in normal human mammary tissue. J Natl Cancer Inst 88:284-290, 1996.

13. Hiller T, Snell L, Watson P. Microdissection/RT-PCR analysis of gene expression. Biotechniques 21: 38-44, 1996.

14. Leygue E, Murphy LC, Kuttenn F, Watson PH. Triple primer-polymerase chain reaction: a new way to quantify truncated mRNA expression. Am J Path 148: 1097-1103, 1996 .

15. Hiller T, Leygue E, Murphy L, Watson P. Quantitation of Estrogen Receptor Clone 4 mRNA Variant in Microdissected Normal and Neoplastic Human Breast Tissues. AACR, April 1996.

16. Singh R, Watson P .Quantitative RT-PCR analysis of c-myc expression in breast cancer. AACR, April 1996.

17. Murphy LC, Hilsenbeck S, Dotzlaw H, Fuqua SAW: Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. Clin Cancer Res 1: 155-159, 1995.

18. Leygue E, Hall R, Dotzlaw H, Watson PH, Murphy LC. Comparison of estrogen receptor-α variant mRNA expression in primary human breast tumors and their matched lymph node metastases. Br J Cancer: in press, 1998. Galleys attached, **Appendix 4.**

19. Coutts A, Leygue E, Murphy LC. Altered estrogen receptor-alpha expression in estrogen independence. Manuscript attached, **Appendix 5.**

20. Coutts A, Davie J, Dotzlaw H, Murphy LC. Estrogen regulation of nuclear matrix-intermediate filament proteins in human breast cancer cells. J Cell Biochem 63: 174-184, 1996.

21. Murphy LC, Leygue E, Dotzlaw H, Coutts A, Lu B, Huang A, Watson PH. Multiple facets of the estrogen receptor in human breast cancer. In, Endocrine Oncology, S Ethier (Ed) Humana Press: in press. Manuscript attached, **Appendix 6.**

22. CouttsA, Murphy LC. Elevated mitogen activated protein kinase activity in estrogen non-responsive human breast cancer cells. Cancer Res 58: 4071-4074, 1998. Reprint attached, **Appendix 7.**

23. Kato S *et al.,* Activation of the estrogen receptor through phosphorylation by mitogen activated protein kinase. Science 270: 1491-1494, 1995.

24. Huang A, Pettigrew N, Watson PH. Immunohistochemical assay for oestrogen receptors in paraffin wax sections of breast carcinoma using a new monoclonal antibody. J Pathol 180: 223-227, 1996.

25. Huang A, Leygue E, Snell L, Murphy LC, Watson PH. Expression of estrogen receptor variant mRNAs and determination of estrogen receptor status in human breast cancer. Am J Pathol 150: 1827-1833, 1997.

26. Huang A, Leygue E, Dotzlaw H, Murphy LC, Watson PH. Estrogen receptor-alpha mRNA variants influence the determination of estrogen receptor status in human breast cancer. Manuscript attached, **Appendix 8.**

27. Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH. Oestrogen receptor variants and mutations in human breast cancer. Annal Med 29: 221-234, 1997.

28. Murphy LC, Dotzlaw H, Leygue E, Coutts A, Watson P. The pathophysiological role of estrogen receptor variants in human breast cancer. J Steroid Biochem Mol Biol 65: 175-180, 1998. Reprint attached, **Appendix 9.**

29. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Altered expression of estrogen receptor-alpha variant mRNAs between adjacent normal breast and breast tumor tissues. Manuscript attached, **Appendix 10.**

30. Dotzlaw H, Leygue E, Watson PH, Murphy LC. Estrogen receptor-beta mRNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. Manuscript attached, **Appendix 11.**

Bibliography of all Publications and Meeting Abstracts Arising from this Grant.

A. Publications:

1. Leygue E, Watson PH, Murphy LC. Estrogen receptor variants in normal human mammary tissue. J Natl Cancer Inst 88:284-290, 1996.
2. Murphy LC, Wang M, Coutts A, Dotzlaw H. Novel Mutations in the Estrogen Receptor mRNA in Human Breast Cancers. J Clin Endocrinol Metab 81: 1420-1427, 1996.
3. Wang M, Dotzlaw H, Fuqua SAW, Murphy LC. A point mutation in the human estrogen receptor gene is associated with the expression of an abnormal estrogen Receptor mRNA containing a 69 novel nucleotide insertion. Breast Cancer Res Treat 44: 145-151, 1997.
4. Leygue E, Murphy LC, Kuttenn F, Watson PH. Triple primer-polymerase chain reaction: a new way to quantify truncated mRNA expression. Am J Path 148: 1097-1103, 1996 .
5. Leygue ET, Huang A, Murphy LC, Watson PH . Prevalence of estrogen receptor variant messenger RNAs in human breast cancer. Cancer Res 56: 4324-4327, 1996.
6. Coutts A, Davie J, Dotzlaw H, Murphy LC. Estrogen regulation of nuclear matrix-intermediate filament proteins in human breast cancer cells. J Cell Biochem 63: 174-184, 1996.
7. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Identification of novel exon-deleted progesterone receptor variant mRNAs in human breast tissues. Biochem Biophy Res Commun 228: 63-68, 1996.
8. Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH. Oestrogen receptor variants and mutations in human breast cancer. Annal Med 29: 221-234, 1997.
9. Huang A, Leygue E, Snell L, Murphy LC, Watson PH. Expression of estrogen receptor variant mRNAs and determination of estrogen receptor status in human breast cancer. Am J Pathol 150: 1827-1833, 1997.
10. Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor-beta in human breast tumors. J Clin Endocrinol Metab 82: 2371-2374, 1997.
11. Murphy LC, Dotzlaw H, Leygue E, Coutts A, Watson P. The pathophysiological role of estrogen receptor variants in human breast cancer. J Steroid Biochem Mol Biol 65: 175-180, 1998.
12. Murphy LC, Dotzlaw H, Leygue E, Douglas D, Coutts A, Watson PH. Estrogen receptor variants and mutations: a review. J Steroid Biochem Mol Biol 62: 363-372, 1997.
13. Murphy LC. Mechanisms of hormone dependence in human breast cancer. In Vivo 12: 95-106, 1998.
14. Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC, Watson PH. Estrogen receptor-beta mRNA variants in human and murine tissues. Mol Cell Endocrinol 138: 199-203, 1998.

15. Leygue E, Hall R, Dotzlaw H, Watson PH, Murphy LC. Comparison of estrogen receptor-α variant mRNA expression in primary human breast tumors and their matched lymph node metastases. Br J Cancer: in press, 1998
16. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Altered estrogen receptor alpha and beta mRNA expression during human breast tumorigenesis. Cancer Res 58: 3197-3201, 1998.
17. Coutts A, Murphy LC. Elevated mitogen activated protein kinase activity in estrogen non-responsive human breast cancer cells. Cancer Res 58: 4071-4074, 1998
18. Leygue E, Dotzlaw H, Lu B, Glor C, Watson PH, Murphy LC. Estrogen receptor beta: mine is longer than yours ? J Clin Endocrinol Metab 83: 3725-3755, 1998.
19. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Altered expression of exon 6 deleted progesterone receptor variant mRNA during human breast tumorigenesis. Br J Cancer: in press.
20. Murphy LC, Leygue E, Dotzlaw H, Coutts A, Lu B, Huang A, Watson PH. Multiple facets of the estrogen receptor in human breast cancer. In, Endocrine Oncology, S Ethier (Ed) Humana Press: in press
21. Leygue E, Dotzlaw H, Lu B, Watson PH, Murphy LC. Estrogen receptor beta: a review. In Current Topics in Steroid Research: in press.

Manuscripts submitted:

1. Dotzlaw H, Leygue E, Watson PH, Murphy LC. Estrogen receptor-beta mRNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins.
2. Leygue E, Dotzlaw H, Watson PH, Murphy LC. Altered expression of estrogen receptor-alpha variant mRNAs between adjacent normal breast and breast tumor tissues.
3. Huang A, Leygue E, Dotzlaw H, Murphy LC, Watson PH. Estrogen receptor-alpha mRNA variants influence the determination of estrogen receptor status in human breast cancer.
4. Coutts A, Leygue E, Murphy LC. Altered estrogen receptor-alpha expression in estrogen independence.

Abstract Presentations.

1. Leygue E, Watson P, Murphy LC (1995) Estrogen receptor variants in normal human mammary tissue. 18th Annual San Antonio Breast Cancer Symposium.
2. Hiller T, Leygue E, Murphy LC, Watson PH (1996) Quantitation of Estrogen Receptor Clone 4 mRNA Variant in Microdissected Normal and Neoplastic Human Breast Tissues. AACR.
3. Leygue E, Huang A, Murphy LC, Watson P (1996) A new approach to investigate estrogen receptor variant mRNA expression in breast tissue. 19th Annual San Antonio Breast Cancer Symposium.

4. Coutts A, Leygue E, Murphy LC (1997) Mechanisms of hormone independence in human breast cancer cells. 88th AACR meeting, San Diego.
5. Dotzlaw H, Leygue E, Watson P, Murphy LC (1997) expression of the estrosy samples. 79th Endocrine Soc Meeting, Minneapolis.
6. Douglas D, Murphy LC (1997) Functional characterization of novel inserted estrogen receptors. 79th Endocrine Soc Meeting, Minneapolis.
7. Dotzlaw H, Leygue E Coutts A, Douglas D, Huang A, Watson P, Murphy LC (1996). Steroid hormone receptor variant mRNAs in human mammary tissues. 3rd Internet World Congress on Biomedical Sciences.
8. Leygue E, Dotzlaw H, Hare H, Watson P, Murphy LC (1997) Expression of estrogen receptor beta variant mRNAs in human breast tumors. 20th Annual San Antonio Breast Cancer Symposium.
9. Murphy LC, Watson PH. (1997) Estrogen receptor variants in human breast cancer. DOD, Breast Cancer Research Program Meeting, Era of Hope. Washington D.C.
10. Murphy LC (invited speaker), Leygue E, Dotzlaw H, Lu B, Coutts A, Huang A, Watson P (1998) Multiple facets of the estrogen receptor in breast cancer. Keystone Symposium on Breast and Prostate Cancer. Copper Mountain, Co.
11. Coutts A, Murphy LC (1998). Elevated MAPK activity and expression in estrogen nonresponsive human breast cancer cells. 89th AACR Annual Meeting, New Orleans.
12. Dotzlaw H, Leygue E, Watson P, Murphy LC (1998). Expression of estrogen receptor alpha and estrogen receptor beta mRNA in primary human breast tumors and matched normal human breast tissue samples. Xth International Congress on Hormonal Steroids, Quebec.
13. Leygue E, Hall R, Dotzlaw H, Watson P, Murphy LC (1998). Conserved patterns of estrogen receptor alpha variant mRNA expression in primary human breast tumors and matched lymph node metastases. 21th Annual San Antonio Breast Cancer Symposium.

List of personnel receiving pay from this effort.

Helmut Dotzlaw
Etienne Leygue Ph.D
D Douglas, PhD.
C Cumins-Leygue
M Wang
H Bergen
M Rempel
M Lafreniere
C Bergen
A Coutts
V Spencer
H Hare
C Glor

APPENDIX 1

ELSEVIER

Molecular and Cellular Endocrinology 138 (1998) 199–203

MCE
Molecular and Cellular Endocrinology

Rapid communication

Estrogen receptor-β mRNA variants in human and murine tissues

B. Lu [a], E. Leygue [a], H. Dotzlaw [a], L.J. Murphy [b], L.C. Murphy [a,*], P.H. Watson [c]

[a] *Department of Biochemistry and Molecular Biology, University of Manitoba, 770 Bannatyne Avenue, R3E 0W3, Winnipeg, Canada*
[b] *Department of Physiology, University of Manitoba, 770 Bannatyne Avenue, R3E 0W3, Winnipeg, Canada*
[c] *Department of Pathology, University of Manitoba, 770 Bannatyne Avenue, R3E 0W3, Winnipeg, Canada*

Accepted 23 January 1998

Abstract

Estrogen receptor (ER)-β mRNA splice variants have been identified in human breast tumors as well as normal human and mouse ovarian, uterine and mammary tissues. In both species transcripts deleted in exons 5 or 6, or 5 + 6 have been characterized by RT-PCR followed by cloning and sequencing. In mouse tissues an ER-β transcript containing 54 nucleotides inserted in frame between exons 5 and 6 was identified. Interestingly, no equivalent of the mouse inserted transcript was detected in any of the four human tissues analyzed.

Keywords: Estrogen receptor-α; Estrogen receptor-β; mRNA variant; Alternative splicing; Human; Mouse

1. Introduction

Recently, the cDNA of a second estrogen receptor, estrogen receptor (ER)-β, was cloned and sequenced from the rat (Kuiper et al., 1996), the human (Mosselman et al., 1996) and the mouse (Tremblay et al., 1997). Northern analysis of RNA isolated from mouse ovary demonstrated the presence of multiple mRNA species for ER-β (Tremblay et al., 1997) suggesting the possibility that variant ER-β proteins might exist. To investigate whether ER-β variant mRNAs might be expressed in human as well as murine tissues, an RT-PCR analysis was undertaken which demonstrated the presence of variant ER-β mRNAs in both species.

2. Materials and methods

2.1. Tissues and RNA extraction

Human breast tumor specimens left-over from steroid receptor assays were obtained from the Manitoba Breast Tumor Bank, and three non-malignant human uterine hysterectomy samples were obtained from the Department of Obstetrics and Gynecology (Health Sciences Centre, Winnipeg, Canada). Total RNA was extracted by the guanidinium thiocyanate/cesium chloride method as previously described (Dotzlaw et al., 1990). Four nonmalignant human ovarian samples from two pre-menopausal and two post-menopausal women were obtained through the Ovarian Tissue Bank (Institut du Cancer de Montreal, Centre de Recherche Louis-Charles Simard, Montreal, Canada). Four normal human breast tissues from reduction mammoplasties of pre-menopausal women were obtained through the Manitoba Breast Tumor Bank. Total RNA from the ovarian and normal

* Corresponding author. Tel.: +1 204 7893233; fax: +1 204 7893900; e-mail: lcmurph@cc.umanitoba.ca

0303-7207/98/$19.00
PII S0303-7207(98)00050-1

breast tissue samples was extracted with Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Mouse uteri and ovaries were obtained from four female mice aged 8–9 weeks, and mammary tissues were obtained from two adult lactating female mice. Total RNA was extracted with Trizol reagent (Gibco/BRL) according to the manufacturer's instructions.

Integrity of RNA was confirmed by denaturing gel electrophoresis as previously described (Murphy and Dotzlaw, 1989).

2.2. RT-PCR and primers

Total RNA (1.5 μg per reaction) was reverse transcribed as previously described (Dotzlaw et al., 1997). One microlitre of this reaction was amplified by PCR incorporating ^{32}P in a final volume of 10 μl, and 4 μl of this reaction separated on 6% denaturing polyacrylamide gels and autoradiographed as previously described (Dotzlaw et al., 1997).

All ER-β exons are defined in this report by analogy to the human ER-β exon structure (Enmark et al., 1997): human primer set one: hER-β exons 4 and 7: hER-β-4 (sense) 5′-GGC CGA CAA GGA GTT GGT A-3′ (priming site in exon 4, nucleotides 762—780 as numbered in Mosselman et al. (1996)); hER-β-7 (antisense) 5′-TCC ATG CCC TTG TTA CTC G-3′ (priming site in exon 7, nucleotides 1262–1280). The PCR conditions were 30 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C.

Human primer set two: hER-β exons 5 and 6: hER-β-5 (sense) 5′-GCT GTT GGA TGG AGG TGT TA-3′ (priming site in exon 5, nucleotides 857–876); hER-β-6 (antisense) 5′-CTT GAA GTA GTT GCC AGG AG-3′ (priming site in exon 6, nucleotides 997–1016). The PCR conditions were 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C.

Mouse primer set one: mER-β exons 4 and 8: mER-β-4 (sense) 5′-CTG AAC AAA GCC AAG AGA-3′ (priming site in exon 4, nucleotides 600–617 as numbered in Tremblay et al. (1997)); mER-β-8 (antisense) 5′-GCT CTT ACT GTC CTC TGT CG-3′ (priming site in exon 8, nucleotides 1417–1436). The PCR conditions were 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C.

Mouse primer set two: mER-β exons 5 and 6: mER-β-5 (sense) 5′-GCT GAT GGT GGG GCT GAT GT-3′ (priming site in exon 5, nucleotides 890–909); mER-β-6 (antisense) 5′-ATG CCA AAG ATT TCC AGA AT-3′ (priming site in exon 6, nucleotides 993–1012). The PCR conditions were 35 cycles of 1 min at 94°C, and 30 s at 60°C.

PCR products from human breast tumors and mouse mammary tissues were subcloned into the cloning vector, pGEM-T Easy (Promega) following the manufacturer's instructions. Double stranded DNA from at least two independent clones from each tissue was sequenced with a T7 Sequencing kit (Pharmacia) following the manufacturer's protocol. All RT-PCRs were carried out at least 2 × for each sample analyzed.

3. Results and discussion

Previously the presence of ER-β mRNA was identified in some human breast tumor samples (Dotzlaw et al., 1997). The RT-PCR analysis employed a primer set which annealed to sequences corresponding to exons 7 and 8 of the human ER-β cDNA (Enmark et al., 1997). Numerous splicing variants of the human ER-α mRNA have been identified to date (Murphy et al., 1997), and it was of interest to determine if similar splice variants could be detected in the ligand binding domain of the ER-β mRNA in human breast tumors. Using a primer set which would anneal to sequences located in exons 4 and 7 of the human ER-β cDNA (Enmark et al., 1997), RT-PCR analyses were undertaken using RNA isolated from four separate human breast tumor samples which had previously been shown to express ER-β mRNA by RT-PCR using an exon 7/8 primer set (Dotzlaw et al., 1997). The results presented in Fig. 1 show the presence of the expected 519 bp wild-type ER-β product, as well as several smaller sized PCR products.

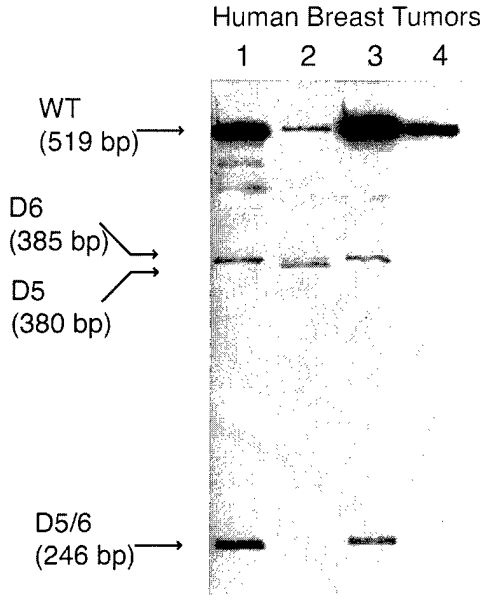


Fig. 1. Detection of wild-type ER-β and ER-β variant mRNAs in human breast tumor tissues. Total RNA extracted from four different human breast tumors (1–4) was reverse transcribed and PCR amplification was carried out using primers located in exons 4 and 7. PCR products migrating at the sizes of 519, 385, 380 and 246 bp were subsequently cloned, sequenced and identified as corresponding to ER-β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5–6-deleted variant (D5/6) cDNAs, respectively.

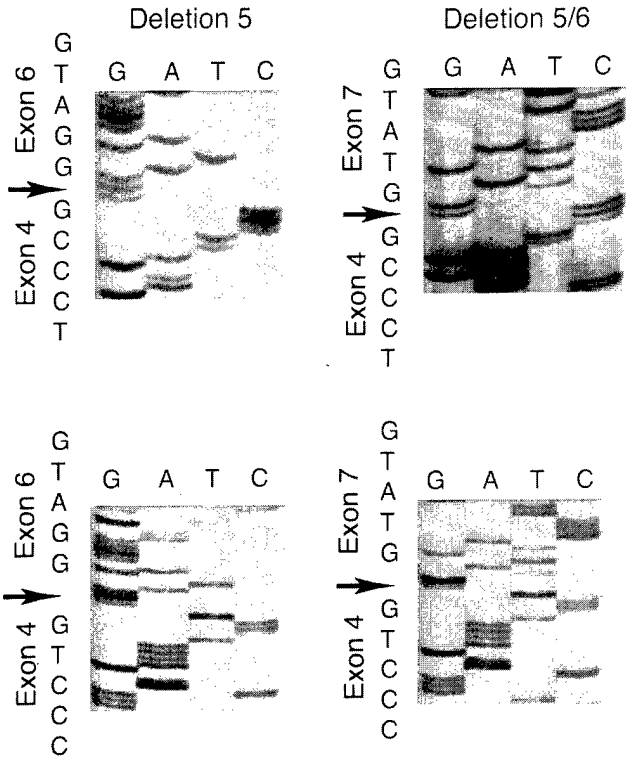


Fig. 2. Sequencing of exon 5-deleted and exon 5/6-deleted ER-β variants in human and murine tissues. Top panels: sequencing of PCR products obtained by amplification of human breast tumor cDNAs using primers in exons 4 and 7 and migrating at the sizes of 380 and 246 bp (Fig. 1) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively. Bottom panels: sequencing of PCR products obtained by amplification of murine breast tissue cDNAs using primers in exons 4 and 8 and migrating at the sizes of 698 and 564 bp (Fig. 3) showed a perfect junction between exon 4 and 6 (deletion 5), and exon 4 and 7 (deletion 5/6), respectively.

Cloning and sequencing of the smaller sized products (Fig. 2) revealed deletions of nucleotides 812–950, 951–1084 and 812–1084 (numbered as in Mosselman et al. (1996)) which are precise exon deletions of exon 5, 6, and 5 + 6, respectively. To determine if such deletions occurred only in human breast tumor tissue, RNA extracted from several normal breast, uterine and ovarian tissue samples was analyzed (Fig. 3, top panel). The same tissues from the mouse were analyzed in parallel, with mouse ER-β primer set located in putative exons 4 and 8 (Fig. 3, bottom panel; all exons, mouse and human, are numbered according to the human ER-β structure (Enmark et al., 1997)). All human tissues analyzed expressed ER-β variant mRNAs similar to those identified in breast tumors. The expected wild-type product of 837 bp was detected in all mouse tissues, as were several smaller sized PCR products. Cloning and sequencing of the 698 and 564 bp fragments identified deletions of nucleotides 829–967 and 829–1101, which by analogy to the human ER-β would be a precise deletion of exon 5 and deletion of exons 5 and 6, respectively (Fig. 2, nucleotides numbered as in Tremblay et al. (1997)). The 703 bp band was found to correspond to an exon 6 deleted ER-β transcript.

These data are the first to support the expression of exon deleted splice variants for ER-β similar to those for ER-α, in both human and murine tissue samples. The exon 5 and 6 deleted splice variants identified in this

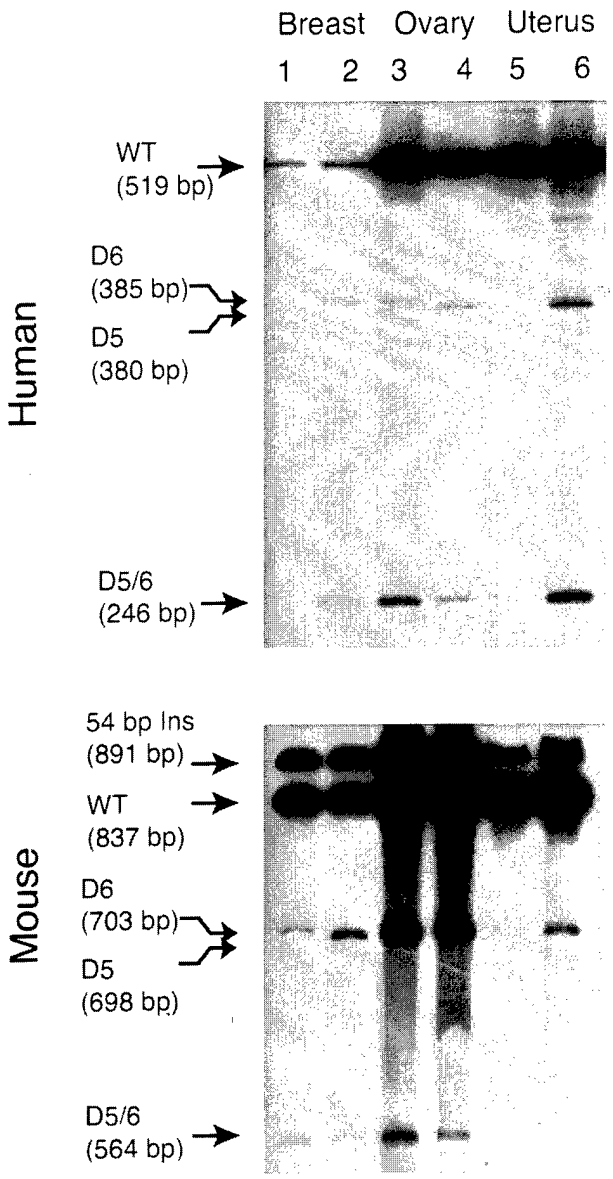


Fig. 3. Detection of wild-type ER-β and ER-β variant mRNAs in normal human and murine tissues. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1–2), ovaries (3–4) and uteri (5–6) was reverse transcribed and PCR amplification was carried out using primers located in exons 4 and 7 (human) or in exons 4 and 8 (mouse). PCR products obtained in human tissues migrated at the sizes of 519, 385, 380 and 246 bp corresponding to ER-β wild-type (WT), exon 6-deleted (D6), exon 5-deleted (D5) and exon 5–6-deleted (D5/6) cDNAs, respectively. PCR products obtained in mouse tissues and migrating at the sizes of 891, 837, 703, 698 and 564 bp were identified as corresponding to a 54 bp inserted ER-β variant (54 bp Ins), ER-β wild-type (WT), exon 6-deleted variant (D6), exon 5-deleted variant (D5) and exon 5–6-deleted variant (D5/6), respectively.

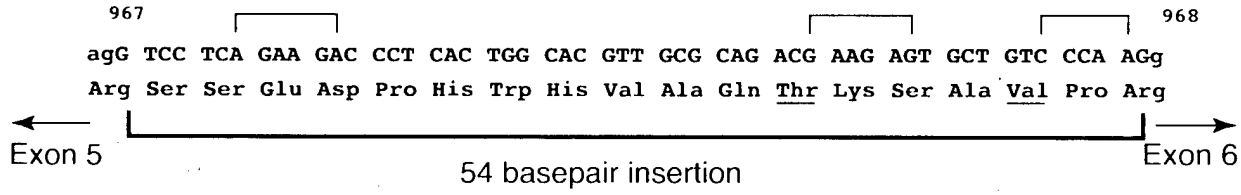


Fig. 4. Insertion (54 bp): nucleotide and amino acid sequences. The sequence of the inserted 54 bp is indicated in upper case letters. Nucleotides corresponding to the published mouse ER-β cDNA sequence, indicated in lower case letter, are numbered according to Dotzlaw et al. (1990). Brackets indicate putative exonic splicing enhancer sequences (Coulter et al., 1997; Otto et al., 1997). Predicted amino acid composition of the insert is shown. The underlined amino acids correspond to the substitutions observed between mouse inserted sequence and the recently described rat inserted sequence (Daffada et al., 1994).

study are out-of-frame and would be expected to encode C-terminally truncated ER-β proteins, which are unlikely to bind ligand. The exon 5 + 6 deleted splice variant is inframe but deleted in 91 amino acids (aa) (274–364 of the mouse and human ER-β (Enmark et al., 1997)), which are within the hormone-binding domain. The putative protein encoded by the exon 5 + 6 deleted variant would also be unlikely to bind ligand.

In contrast to what was observed in human tissues, all murine tissues analyzed presented a prominent ER-β 891 bp PCR product which was larger than the expected wild-type ER-β product of 837 bp (Fig. 3, bottom panel). Sequencing of the larger PCR product revealed an insertion of 54 nucleotides between nucleotides 967 and 968 (Tremblay et al., 1997), which is precisely inserted between the splice junction of exons 5 and 6. Identical results were obtained when the starting RNA samples were enriched for polyadenylated transcripts using oligo-dT attached to magnetic beads (data not shown), suggesting that the inserted transcript represented an authentic mRNA species. The sequence of this insertion is shown in Fig. 4. This insertion is inframe and the predicted amino acids are shown in Fig. 4 also. While this work was in progress the presence of a 54 bp inserted ER-β transcript in rat tissues was published (Chu and Fuller, 1997). The sequence of the 54 nucleotide insertion in the murine ER-β transcript is identical to that published for the rat except for a T → C change at nucleotide position 36 (1 = start of the 54 nucleotide insert), which would result in a Met → Thr substitution in the mouse protein, and a C → T change at position 48 which would result in an Ala → Val substitution in the mouse protein.

Because the initial screening of human tissues using the exon 4/7 primer set failed to reveal an analogous human ER-β transcript containing an insertion between exons 5 and 6, reanalysis of human and mouse tissues was undertaken using primer sets located in exons 5 and 6 of either the human or the mouse ER-β. While the inserted ER-β transcript was easily detected as a 177 bp PCR product in all murine tissues analyzed, only the expected 160 bp PCR product corresponding to the human wild-type ER-β mRNA was detected in the normal human tissues (Fig. 5), and an inserted ER-β variant was similarly not detected in ten human ER-β RNA positive breast tumor samples (Dotzlaw et al., 1997) (data not shown). The data shown in Fig. 5 suggest that the inserted transcript is predominant in both mouse mammary gland and uterus, while similar levels of each transcript occur in the mouse ovary. It should be noted that the primers used in this latter analysis (Fig. 5) were designed to detect the mouse ER-β wild-type and the inserted transcript. Thus only two competing PCR products are obtained, and measurement of relative expression using such an approach has been validated previously (Daffada et al., 1994; Leygue et al., 1996). This is in contrast to the primer set used in Fig. 3 which detects up to five PCR products, is designed for the study of relative patterns of expression and is unlikely to accurately reflect the relative expression of any two individual species under such conditions.

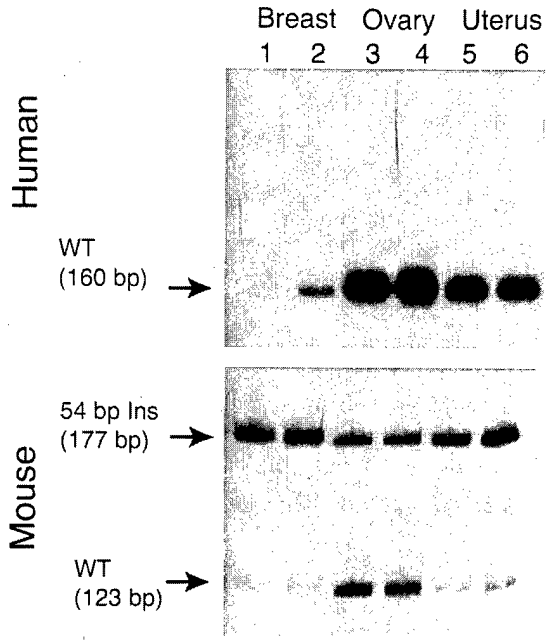


Fig. 5. Amplification of human and murine cDNAs using exon 5 and 6 primers. Total RNA extracted from normal human (top panel) or mouse (bottom panel) breast tissues (1–2), ovaries (3–4) and uteri (5–6) was reverse transcribed and PCR amplification was carried out using primers located in exons 5 and 6. The PCR product obtained in human tissues migrated at the size of 160 bp corresponding to ER-β wild-type (WT). PCR products obtained in mouse tissues migrated at the sizes of 177 and 123 bp, corresponding to a 54 bp inserted ER-β variant (54 bp Ins) and ER-β wild-type (WT), respectively.

A possible mechanism associated with the frequent inclusion of the inserted sequences in mouse ER-β transcripts may be the presence of several putative exonic splicing enhancer sequences within the insertion sequences (Cooper and Mattox, 1997; Coulter et al., 1997). Both purine rich motifs and A/C-rich splicing enhancer sequences are present (brackets in Fig. 4). The frequent inclusion of the inserted sequences in mouse ER-β transcripts, and their presence in several tissues at comparable levels with the wild-type transcript suggest that the protein encoded by the inserted transcript has a functional role, at least in the mouse and rat (Chu and Fuller, 1997). The putative function of a protein encoded by the inserted mouse ER-β transcript is unknown, and since the insertion is in the middle of the ligand binding domain, it may either disrupt binding completely or result in a different ligand binding specificity and/or affinity. Further, the insertion may effect the three dimensional structure of the ER-domain such that alterations in dimerization, transactivation and interaction with co-regulators may also occur. This could result in the inserted ER-β having a regulatory function on the wild-type ER-β as previously suggested (Chu and Fuller, 1997), or may completely alter its ability to heterodimerize and affect the activity of ER-α (Cowley et al., 1997). The lack of detection of a similar inserted ER-β transcript in human tissues may be due to hormonal differences at the time of tissue collection between the mouse and human subjects, or to a real species difference in alternative splicing. In the latter instance differences in alternative splicing between mouse and human with regard to a steroid hormone receptor have been previously documented (Oakley et al., 1996; Otto et al., 1997). Similarly, inserted sequences within the ligand binding domain of ER-α have also been reported (Murphy et al., 1996). In contrast with the inserted ER-β mRNA, the inserted ER-α mRNA was detected in one human breast tumor sample, and was due to a point mutation in one allele of the human ER-α gene present in the breast tumor (Wang et al., 1997).

In summary, in this report deletion splice variants of ER-β have been characterized for the first time in several mouse and human tissues, both normal and neoplastic. An inserted splice variant of the ER-β mRNA, previously identified in the rat, has been confirmed in several mouse tissues. This inserted variant was undetected in any human tissues analyzed, suggesting species specific differences in its expression.

Acknowledgements

This work was supported by the US Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). L.C.M. is a Medical Research Council of Canada (MRC) Scientist, P.H.W. is a MRC clinician-scientist, E.L. is a recipient of a USAMRMC Postdoctoral Fellowship. The authors thank Heidi Hare for excellent technical assistance.

References

Chu, S., Fuller, P., 1997. Identification of a splice variant of the rat estrogen receptor β gene. Mol. Cell. Endocrinol. 132, 195–199.

Cooper, T., Mattox, W., 1997. The regulation of splice-site selection, and its role in human disease. Am. J. Hum. Genet. 61, 259–266.

Coulter, L., Landree, M., Cooper, T., 1997. Identification of a new class of exonic splicing enhancers by in vivo selection. Mol. Cell. Biol. 17, 2143–2150.

Cowley, S., Hoare, S., Mosselman, S., Parker, M., 1997. Estrogen receptors alpha and beta form heterodimers on DNA. J. Biol. Chem. 272, 19858–19862.

Daffada, A.A., Johnston, S.R., Nicholls, J., Dowsett, M., 1994. Detection of wild-type and exon 5 deleted splice variant oestrogen receptor (ER) mRNA in ER-positive and -negative breast cancer cell lines by reverse transcription/polymerase chain reaction. J. Mol. Endocrinol. 13, 265–273.

Dotzlaw, H., Miller, T., Karvelas, J., Murphy, L.C., 1990. Epidermal growth factor gene expression in human breast cancer biopsy samples: relationship to estrogen and progesterone receptor gene expression. Cancer Res. 50, 4204–4208.

Dotzlaw, H., Leygue, E., Watson, P.H., Murphy, L.C., 1997. Expression of estrogen receptor-beta in human breast tumors. J. Clin. Endocrinol. Metab. 82, 2371–2374.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., Gustafsson, J.A., 1997. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J. Clin. Endocrinol. Metab. 82, 4258–4265.

Kuiper, G., Enmark, E., Pelto-Huikko, M., Nilsson, S., Gustafsson, J.A., 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA 93, 5925–5930.

Leygue, E., Watson, P.H., Murphy, L.C., 1996. Estrogen receptor variants in normal human mammary tissue. J. Natl. Cancer Inst. 88, 284–290.

Mosselman, S., Polman, J., Dijkema, R., 1996. ER-beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 392, 49–53.

Murphy, L.C., Dotzlaw, H., 1989. Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. Mol. Endocrinol. 3, 687–693.

Murphy, L.C., Wang, M., Coutts, A., Dotzlaw, H., 1996. Novel mutations in the estrogen receptor messenger RNA in human breast cancers. J. Clin. Endocrinol. Metab. 81, 1420–1427.

Murphy, L.C., Leygue, E., Dotzlaw, H., Douglas, D., Coutts, A., Watson, P., 1997. Oestrogen receptor variants and mutations in human breast cancer. Ann. Med. 29, 221–234.

Oakley, R., Sar, M., Cidlowski, J., 1996. The human glucocorticoid receptor isoform: expression, biochemical properties, and putative function. J. Biol. Chem. 271, 9550–9559.

Otto, C., Reichardt, H., Schutz, G., 1997. Absence of glucocorticoid receptor-beta in mice. J. Biol. Chem. 272, 26665–26668.

Tremblay, G., Tremblay, A., Copeland, N., Gilbert, D., Jenkins, N., Labrie, F., Giguere, V., 1997. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol. Endocrinol. 11, 353–365.

Wang, M., Dotzlaw, H., Fuqua, S., Murphy, L.C., 1997. A point mutation in the human estrogen receptor gene is associated with the expression of an abnormal estrogen receptor mRNA containing a 69 novel nucleotide insertion. Breast Cancer Res. Treat. 44, 145–151.

APPENDIX 2

[CANCER RESEARCH 58, 3197–3201, August 1, 1998]

Advances in Brief

Altered Estrogen Receptor α and β Messenger RNA Expression during Human Breast Tumorigenesis[1]

Etienne Leygue,[2] Helmut Dotzlaw, Peter H. Watson, and Leigh C. Murphy

Departments of Biochemistry and Molecular Biology [E. L., H. D., L. C. M.] and Pathology [P. H. W.], University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, R3E OW3 Canada

Abstract

Using a multiplex reverse transcription-PCR assay, we compared the relative expression of estrogen receptor (ER) α and ER-β mRNA between adjacent samples of normal breast tissue and matched primary breast tumors obtained from 18 different patients. Within this cohort, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. No differences in the ratio of ER-α:ER-β expression were observed in the ER-negative cohort. However, in the ER-positive cohort, a significantly ($P < 0.02$) higher ER-α:ER-β ratio was observed in the tumor compared with that of the normal tissue component. Our data revealed that the increase in the ER-α:ER-β ratio was due primarily to a significant ($P < 0.05$) increase in ER-α mRNA expression in conjunction with a lower ER-β mRNA expression in the tumor compared with that of the normal compartment in some, but not all, ER-positive cases. These results suggest that the role of ER-α- and ER-β-driven pathways and/or their interaction change during breast tumorigenesis.

Introduction

Until recently, estrogen action was thought to be mediated principally through a single ER[3], ER-α, a member of the steroid/thyroid/retinoic acid receptor superfamily (1). As with other members of the family, the ER-α protein consists of several structural and functional domains (A–F). The NH_2-terminal transactivation function (AF-1) of the receptor is located within the A–B regions, whereas the DNA binding, the ligand-binding domain, and the second transactivation function (AF-2) reside in the C and E regions of the molecule, respectively (2). Upon ligand binding, conformational changes occur, and two ER-α molecules complexed with the hormone bind specifically to EREs located upstream of target genes. Interactions between ER-α and accessory proteins ultimately lead to the modification of the transcription of these genes (3). Similarly, the ER-α/estrogen complex can interact with c-*fos*/c-*jun* complexes to activate the transcription of target genes through activator protein 1 enhancer elements (4). Recently, a second ER, ER-β, was identified in the human, rat, and mouse (5–7). ER-β shares a similar structural and functional composition with ER-α and was also shown to activate the transcription of target genes through similar EREs (5, 8). However, differential activation of ER-α and ER-β by the antiestrogen 4-hydroxytamoxifen has been shown with ERE-regulated reporter genes (9), and the two ERs also show differential activation of activator protein 1-regulated reporter genes (10), suggesting different roles for these two receptors. In addition, because heterodimerization of ER-α and ER-β has been demonstrated *ex vivo*, putative cross-talk of the two signaling pathways has been suggested (11). The tissue-specific expression of ER-α and ER-β, although not identical, shows some overlap. It has therefore been speculated that estrogen action in a given tissue may depend on the relative expression of these two receptors.

The recent demonstration of ER-β expression in both human breast tumors (12–14) and normal human breast tissue (14, 15) suggests that the well-documented role of estrogen in breast tumorigenesis (16) may also involve both receptors. To determine whether altered expression of these two receptors might occur during breast tumorigenesis, we have compared the relative expression of ER-α and ER-β mRNAs in normal human breast tissues adjacent to matched primary breast tumors.

Materials and Methods

Human Breast Tissues and Cell Line. Eighteen cases were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block was determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks, as described previously (17). The presence of normal ducts and lobules (median $n = 6$; range, 2–13) as well as the absence of any atypical lesion was confirmed in all normal tissue specimens. Seven tumors were ER negative (ER < 3 fmol/mg protein), with progesterone receptor values ranging from 2.2–11.2 fmol/mg protein, as measured by the ligand binding assay. Eleven tumors were ER positive (range, 3.5–159 fmol/mg protein), with progesterone receptor values ranging from 5.8–134 fmol/mg protein. These tumors spanned a wide range of grade (grade, 5–9), which was determined using the Nottingham grading system. For all normal and tumor samples, the percentage of epithelial cells, stromal component, and fat has been estimated by observation of the adjacent paraffin-embedded sections. For normal tissue, the median of the percentage of epithelial cells, stroma, and fat observed within the sections was 10 (range, 5–30%), 50 (range, 5–85%), and 40% (range, 5–90%), respectively. For tumor tissues, the median of the percentage of epithelial tumor cells, normal epithelial cells, stroma, and fat within the sections was 40 (range, 20–60%), 2.5 (range, 0–10%), 37.5 (range, 20–65%), and 20% (range, 10–50%), respectively. Three tumors (T1, T2, and T3) shown in a previous study (12) to express low ER-β/high ER-α, high ER-β/low ER-α, and high ER-β/high ER-α mRNA levels, respectively, were used to validate a multiplex RT-PCR that was designed to determine the relative expression of ER-α:ER-β mRNA. MDA-MB-231 cells were grown and harvested, and the cell pellets were stored at −70°C, as described previously (12). Total RNA was extracted from 20 μm of frozen tissue sections (15- and 5-μm sections for normal and tumor breast tissue, respectively) or cell pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. One μg of total RNA was reverse-transcribed in a final volume of 25 μl as described previously (12).

Primers and PCR Conditions. The primers used consisted of ER-β-U primer [5′-GTCCATCGCCAGTTATCACATC-3′ (sense), located in ER-β 130–151] and ER-β-L primer [5′-GCCTTACATCCTTCACACGA-3′ (anti-

Received 4/23/98; accepted 6/15/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by grants from the Canadian Breast Cancer Research Initiative and the U. S. Army Medical Research and Material Command. The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada. P. H. W. is a Medical Research Council of Canada clinician-scientist. L. C. M. is a Medical Research Council of Canada scientist. E. L. is a recipient of a U. S. Army Medical Research and Material Command Postdoctoral Fellowship.

[2] To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, R3E OW3 Canada. Phone: (204) 789-3812; Fax: (204) 789-3900; E-mail: eleygue@cc.umanitoba.ca.

[3] The abbreviations used are: ER, estrogen receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERE, estrogen-responsive elements.

sense), located in ER-β 371–352]. The nucleotide positions given correspond to published sequences of human ER-β cDNA (5). The other pair of primers used consisted of ER-α-U primer [5′-TGTGCAATGACTATGCTTCA-3′ (sense), located in ER-α 792–811] and ER-α-L primer [5′-GCTCTTCCTC-CTGTTTTTA-3′ (antisense), located in ER-α 940–922]. The nucleotide positions given correspond to published sequences of human ER-α cDNA (1). PCR amplifications were performed, and PCR products were analyzed as described previously, with minor modifications (12). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl in the presence of 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 2 ng/μl ER-α-U/ER-α-L and/or 4 ng/μl ER-β-U/ER-β-L, and 0.3 unit of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed *GAPDH* cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide as described previously (12). PCR products were subcloned and sequenced as described previously (12).

Multiplex PCR Validation. Total RNA was extracted from MDA-MB-231 cells previously shown to express very low ER-α but higher ER-β mRNA levels (12) and from tumors T1, T2, and T3, the characteristics of which are described above. In the first series of experiments, six cDNA preparations were prepared that contained varying percentages of MDA-MB-231 and tumor T1 cDNA by mixing 10, 8, 6, 4, and 0 μl of MDA-MB-231 cDNA with 0, 2, 4, 6, 8, and 10 μl of tumor T1 cDNA (0, 20, 40, 60, 80, and 100% T1 cDNA, respectively). The same experiment was reproduced using a 10-fold dilution of these six cDNA preparations. A second series of experiments was performed in which the six cDNA preparations contained a constant amount of MDA-MB-231 cDNA (5 μl) and 0, 1, 2, 3, 4, and 5 μl of T1 cDNA in a final volume of 10 μl (0, 10, 20, 30, 40, and 50% T1 cDNA, respectively). A third series of experiments contained six cDNA preparations in which the amount of T1 cDNA was held constant (5 μl) with increasing amounts of 0, 1, 2, 3, 4, and 5 μl of MDA-MB-231 cDNA in a final volume of 10 μl (0, 10, 20, 30, 40, and 50% MDA-MB-231 cDNA, respectively). Finally, 1 μl of T1, T2, and T3 cDNA was amplified independently for 22, 26, 30, and 34 cycles. In every case, PCR products were separated on 6% polyacrylamide gels containing 7 M urea. After electrophoresis, the gels were dried and autoradiographed. Signals were quantified by excision of the appropriate bands, the addition of 5 ml of scintillant (ICN Pharmaceuticals, Inc., Irvine, CA), and counting in a scintillation counter (Beckman Instruments).

Quantification and Statistical Analyses. To quantitate ER-α mRNA expression relative to ER-β mRNA expression, coamplification of ER-α and ER-β cDNAs was performed using the multiplex PCR described above. Quantification of the signals was carried out by the excision of the bands corresponding to ER-α and ER-β cDNAs, the addition of scintillant, and scintillation counting. Three independent PCRs were performed. To control for variations between experiments, a value of 100% was assigned to the highest signal measured in each set of PCR experiments, and all signals were expressed as a percentage of this signal. Indeed, the same tissue sample showed the highest signal in all experiments. For each sample, the ER-α:ER-β ratio was calculated. Differences between averages of log(ER-α:ER-β) obtained for matched normal and tumor compartments were tested using the two-tailed Wilcoxon signed rank test.

Evaluation of ER-α and ER-β mRNA expression was performed by independent amplification of both ER-α and ER-β cDNA, *i.e.* using ER-α- or ER-β-specific primers. Two independent PCRs were performed. To control for variations between experiments, all signals were expressed as a percentage of the highest signal observed. In parallel, *GAPDH* cDNA was amplified, and after analysis of the PCR products on prestained agarose gels, the signals were quantified by scanning using NIH Image 161/ppc software. Each *GAPDH* signal was also expressed as a percentage of the highest signal observed in the experiment. The average of ER-α and ER-β signals was then expressed as a percentage of the *GAPDH* signal. Differences between matched normal and tumor elements were tested using the two-tailed Wilcoxon signed rank test. Correlations were tested by calculation of the Spearman coefficient (*r*).

Results

Multiplex PCR as an Approach to Determine the Relative Expression of ER-α and ER-β. To determine the relative expression of ER-α and ER-β mRNA within any individual sample, we used a multiplex PCR assay. In this assay, two set of primers are added to each individual PCR, thus allowing the coamplification of both ER-α and ER-β cDNA in a single tube and therefore eliminating variation introduced due to differences in cDNA loading. To determine whether the results obtained from the multiplex PCR assay directly reflected the initial ER-α:ER-β cDNA ratio, a series of preliminary experiments was conducted. In these experiments, four different cDNA preparations were used. MDA-MB-231 cells, breast tumor T1, breast tumor T2, and breast tumor T3 had been previously shown to contain high ER-β/low ER-α, very low ER-β/high ER-α, high ER-β/low ER-α, and high ER-β/high ER-α mRNA levels, respectively (12). The first experiment consisted of the multiplex amplification of spiked cDNA preparations containing various percentages of MDA-MB-231 and T1 cDNAs (Fig. 1). As shown in Fig. 1*A*, the PCR signal corresponding to ER-β in MDA-MB-231 cells decreased with decreasing input of MDA-MB-231 cDNA, and the ER-α signal increased with increasing input of T1 cDNA. Quantification of the ER-α:ER-β ratio signals was plotted as a function of the percentage of T1 cDNA input (Fig. 1*B*). A direct relationship was found. Similar results were obtained using a 10-fold dilution of the cDNA preparations (data not shown). Using a constant amount of MDA-MB-231 cDNA plus or minus increasing amounts of T1 cDNA (containing primarily ER-α cDNA), a linear increase in the ER-α:ER-β ratio with increasing ER-α input (T1 cDNA) was found (Fig. 2, *A* and *B*). An inverse but linear relationship was obtained using a constant amount of T1 cDNA and increasing amounts of MDA-MB-231 cDNA input (data not shown). Finally, the rank of ER-α:ER-β ratios in T1, T2, and

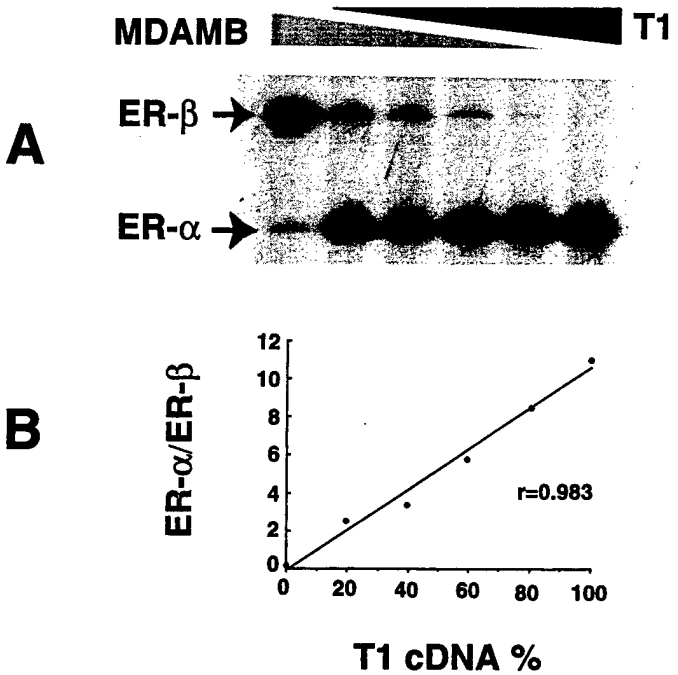


Fig. 1. Multiplex amplification of MDA-MB-231 (low ER-α/high ER-β content) and tumor T1 (low ER-β/high ER-α content) cDNA mixed preparations. An aliquot of solutions containing an increasing amount of tumor T1 cDNA and a decreasing amount of MDA-MB-231 cDNA was prepared and amplified by PCR using ER-α- and ER-β-specific primers in a single tube, and PCR products were separated on an acrylamide gel as specified in "Materials and Methods." *A*, autoradiograph of the gel. *B*, the ER-α:ER-β ratio is expressed as a function of the percentage of tumor T1 cDNA contained in the initial preparation.

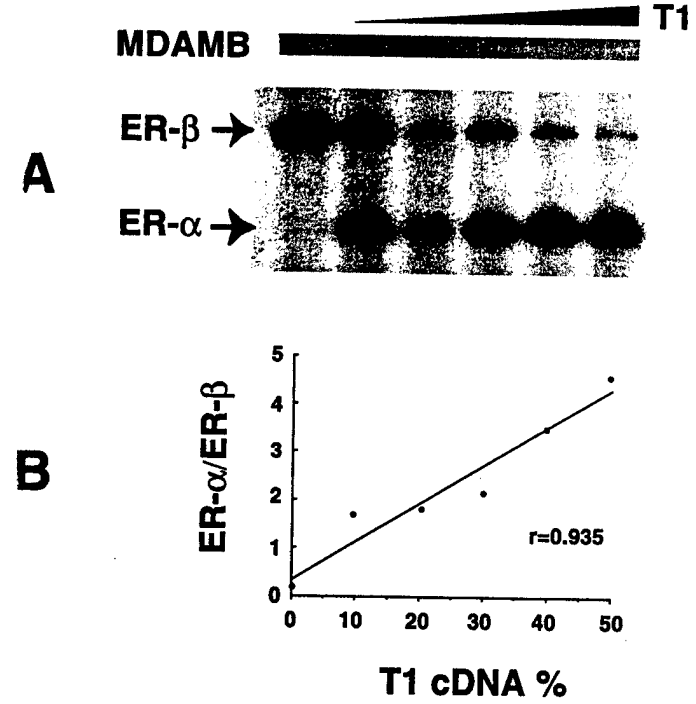


Fig. 2. Multiplex amplification of a constant amount of MDA-MB-231 (low ER-α/high ER-β content) cDNA and an increasing amount of tumor T1 (low ER-β/high ER-α content) cDNA. An aliquot of solutions containing a constant amount of MDA-MB-231 cDNA and an increasing amount of tumor T1 cDNA was prepared, amplified by multiplex PCR, and separated on an acrylamide gel as specified in "Materials and Methods." *A*, autoradiograph of the gel. *B*, the ER-α:ER-β ratio signals is expressed as a function of the percentage of tumor T1 cDNA contained in the initial preparation.

T3 using the multiplex PCR assay was not affected by the number of cycles used for the PCR over a range of 22–34 cycles. The ranking was similar to that deduced using several independent PCR determinations of the ER-α and ER-β mRNA levels, *i.e.* T1 ER-α:ER-β > T3 ER-α:ER-β > T2 ER-α:ER-β (data not shown). Multiplex PCR performed under the described conditions therefore seemed to be a reliable method with which to compare small tissue samples for their relative expression of ER-α and ER-β mRNA.

Comparison of the Relative Expression of ER-α:ER-β mRNA in Adjacent Normal Breast Tissue and Matched Primary Breast Tumors. To determine whether alterations may occur in the contribution of ER-α and ER-β signaling during breast tumorigenesis, the relative expression of ER-α and ER-β mRNA was measured in matched normal and primary tumor tissues from 18 different patients. Within the cohort of tumors studied, 7 tumors were ER negative, and 11 tumors were ER positive, as determined by the ligand binding assay. Total RNA was extracted from the frozen tissue sections and analyzed by multiplex RT-PCR. Examples of the results obtained are shown in Fig. 3*A*. In both normal and tumor compartments, two PCR products migrating at an apparent size of 242 and 149 bp were obtained. These PCR products were identified by cloning and sequencing to correspond to ER-β and ER-α cDNA, respectively. ER-α and ER-β signals obtained in three independent multiplex PCRs were quantified as described in "Materials and Methods." The ER-α:ER-β ratio was calculated for each sample, and the results for each matched sample are presented in Fig. 4*A*. Considering all cases together, no significant change in the ER-α:ER-β ratio was observed between normal and tumor compartments. The cases were then divided into two groups based on the ER positivity of their tumors. Once again,

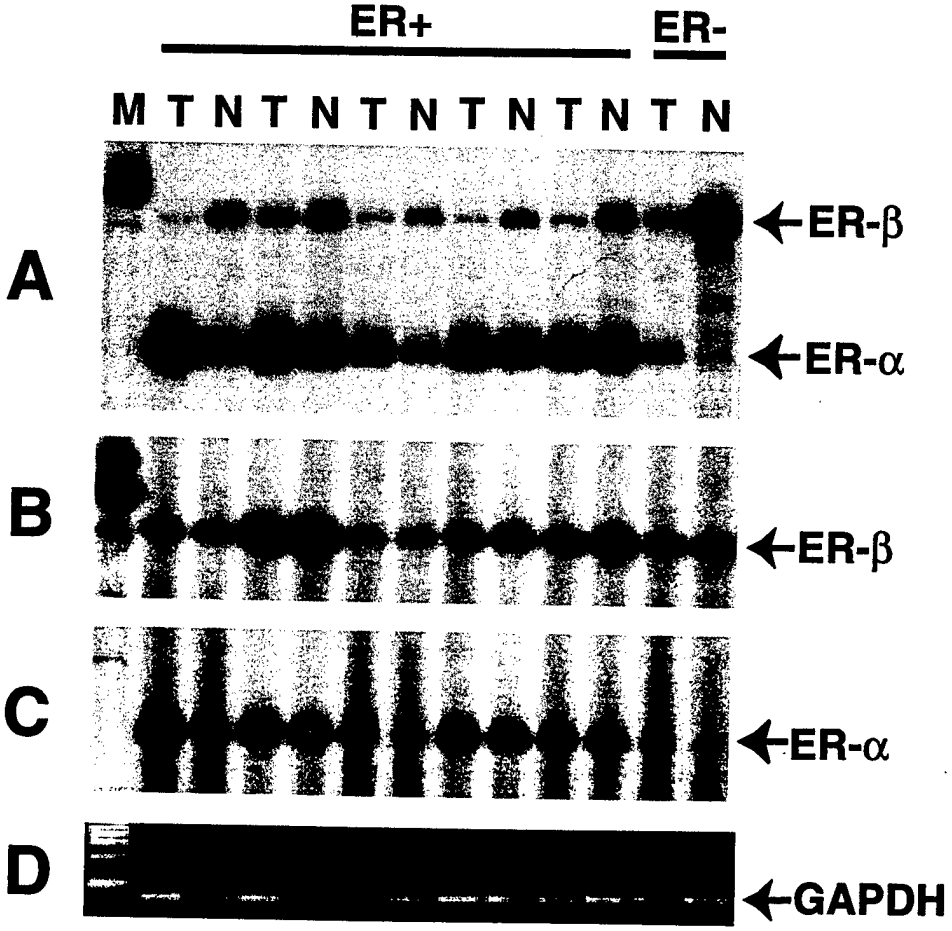


Fig. 3. RT-PCR analysis of ER-α and ER-β expression within matched normal and tumor compartments of human breast tumors. Total RNA was extracted from matched normal (*N*) and tumor (*T*) compartments of ER-positive (*ER+*) or ER-negative (*ER−*) tumors, as determined by ligand binding assay. Corresponding cDNA was amplified, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." PCR was performed using: *A*, ER-α and ER-β primer sets in a single tube (multiplex PCR); *B*, ER-β-specific primers only; *C*, ER-α-specific primers only. *D*, for each sample, *GAPDH* cDNA was amplified in parallel, and PCR products were separated on an agarose gel, as described in "Materials and Methods." *M*, molecular weight marker (ϕx174 RF DNA/*Hae*III fragments; Life Technologies, Inc.).

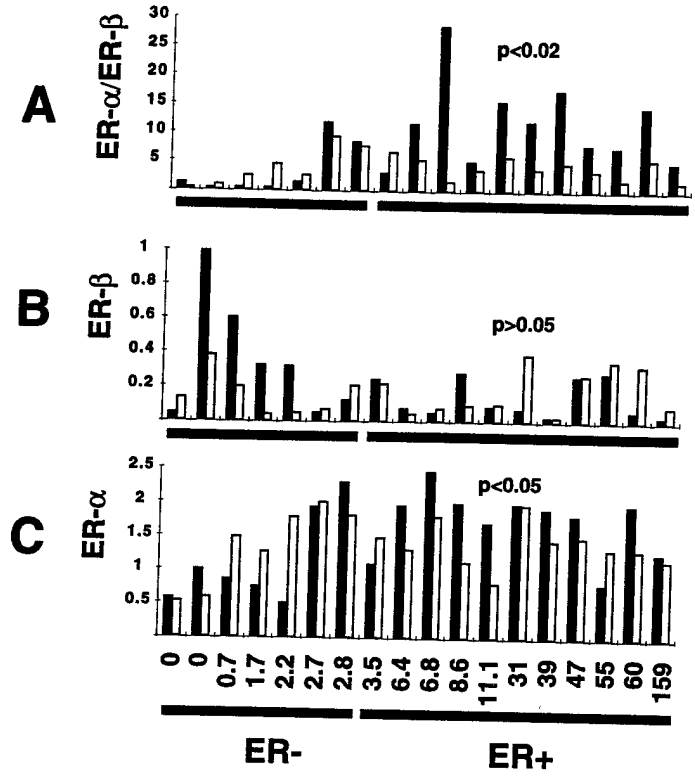


Fig. 4. Quantification of ER-α and ER-β expression within matched normal and tumor compartments of human breast tumors. Total RNA extracted from matched normal (□) and tumor (■) compartments of ER-positive (*ER+*) or ER-negative (*ER−*) tumors was reverse-transcribed and PCR-amplified, and PCR products were separated on an acrylamide gel, as described in "Materials and Methods." Signals have been quantified and normalized, as indicated in "Materials and Methods." *A*, ER-α:ER-β ratio obtained after multiplex PCR. *B*, ER-β signals obtained after PCR was performed using ER-β-specific primers only. *C*, ER-α signals obtained after PCR was performed using ER-α-specific primers only. ER status of the tumor component, as assessed by ligand binding assay, is indicated (fmol/mg protein). Differences between matched normal and tumor compartments in ER-positive cases were tested using the two-tailed Wilcoxon signed rank test.

within the ER-negative cohort, no difference in the ER-α:ER-β ratio was seen between normal tissue and matched tumors. In contrast, in the ER-positive tumor group, a significant increase (two-tailed Wilcoxon signed rank test, $P < 0.02$) in the ER-α:ER-β ratio was observed in the tumor compartment compared with that of the normal compartment. It should be stressed that a significant correlation was found between the ER-α:ER-β ratio observed in the tumor compartment and ER status by binding (Spearman $r = 0.603$; $P < 0.01$).

Independent Measurement of ER-α and ER-β mRNA Expression within Matched Normal and Tumor Compartments. The observed increase in the ER-α:ER-β mRNA ratio of ER-positive breast tumors *versus* matched normal tissue could result from a decrease in the absolute levels of ER-β mRNA and/or an increase in the absolute ER-α mRNA levels within the tumor compartment relative to the matched normal tissue. To distinguish between these possibilities, the ER-β and ER-α mRNA levels were determined individually in each sample by RT-PCR, using either ER-β-specific primers or ER-α-specific primers. Examples of the results obtained are shown in Fig. 3, *B* and *C*, respectively. In parallel, amplification of the ubiquitously expressed *GAPDH* cDNA was also performed (Fig. 3*D*). For each sample, the ER-β and ER-α cDNA signals were quantified, and the average of signals obtained in two independent PCRs was normalized to the *GAPDH* signal, as described in "Materials and Methods." The results are shown in Fig. 4, *B* and *C*, for ER-β and ER-α, respectively. No significant change in ER-β or ER-α mRNA expression was observed between the normal and tumor compartments within the ER-negative cases. Although the difference did not reach statistical significance ($P > 0.05$), ER-β mRNA expression was higher in the normal compartment *versus* the matched tumor component in 8 of 11 (72%) ER-positive cases. A significant (two-tailed Wilcoxon-signed rank test, $P < 0.05$) increase in ER-α mRNA expression was measured in the tumor compartment of ER-positive tumors compared with that of the matched normal tissues. ER-α and ER-β signals observed in normal or tumor compartments did not correlate with the cellular composition of the section analyzed, *i.e.* percentage of normal epithelial cells, tumor epithelial cells, stroma, or fat (data not shown). One should note that although not statistically significant, trends toward an association between ER status by binding and ER-α (Spearman $r = 0.397$; $P = 0.10$) and ER-β (Spearman $r = -0.4254$; $P = 0.07$) have been observed.

Discussion

The discovery of the expression of a second ER in both normal and neoplastic human mammary tissues (12–15), together with the known perturbations of estrogen and antiestrogen sensitivity during breast tumorigenesis and breast cancer progression (16, 18–21), necessitates an investigation of the function of ER-β in human mammary tissue and a reevaluation of the estrogen signal transduction system in these tissues. We have used a multiplex assay in which ER-α and ER-β cDNA are amplified in the same reaction to investigate the relative expression of ER-α and ER-β mRNAs between adjacent samples of normal breast tissue and matched primary breast tumors. The choice of an RT-PCR-based approach to address the question of the relative expression of both receptors has been dictated by several parameters: (*a*) the absence of any publication to date using antibodies to detect ER-β protein in human breast tissue suggests that reliable antibodies are not yet available for this purpose; and (*b*) the expression of ER-β mRNA is relatively low in breast tissue, as demonstrated by the time needed to observe a signal in epithelial human breast cells in *in situ* hybridization studies (14) and by much weaker signals obtained, compared with ER-α, when analyzing breast tissue samples by RNase protection assay.[4] The multiplex PCR assay developed here seems to be a reliable method with which to compare tissue samples for their relative expression of ER-α and ER-β mRNA. It should be stressed, however, that despite the good overall correlation coefficient observed, samples with an ER-α:ER-β ratio of <2 may be less reliably compared with each other (Fig. 2). This could possibly be a limitation of the multiplex approach, which would likely have a higher impact when comparing ER-negative tumors, in which ER-α is known to be weakly expressed. Such a limitation of multiplex PCR analysis of genes expressed at very low levels has previously been reported and may be circumvented by increasing cDNA input (22).

Our data show that in the cohort of patients whose tumors are ER positive by ligand binding, the ratio of ER-α:ER-β is significantly higher in breast tumors than it is in adjacent normal tissues. This difference seems mainly due to an up-regulation of ER-α mRNA levels within the tumor compartment. This observation is in agreement with previous published data showing a generally higher expression of ER-α detected immunohistochemically in ER-positive breast tumors than in normal breast tissue (see Ref. 23 and references herein). However, it is possible that down-regulation of ER-β expression in the tumor tissue may also contribute to the altered ratio in some tumors. Although the difference did not reach statistical significance, 72% of the ER-positive cohort showed a trend in which ER-β expression was lower in the tumor when compared with the normal compartment. The study of larger numbers of cases will be necessary to confirm this trend. Similarly, although no correlations have been

[4] Unpublished observation.

observed between the expression of ER-α and ER-β assessed by targeted PCR and the cellular composition of the sections analyzed, one cannot exclude the possibility that such relationships might exist. The study of a larger number of samples will also clarify this issue. If these RNA studies are paralleled at the protein level, then our data suggest that a significant change in the ratio of these two ERs occurs between normal and neoplastic breast tissues. This would further suggest that the contribution of ER-α- and ER-β-driven pathways and/or their interactions changes in conjunction with breast tumorigenesis. The hypothesis that such changes in ER-α and ER-β signaling pathways may occur during tumorigenesis is also supported by the recent observations of Brandenberger *et al.* (24). These authors showed that ER-α mRNA expression is equal or slightly higher in ovarian cancer tissues compared with normal ovary tissues, and ER-β mRNA expression is decreased in ovarian tumor tissue. The measurement of the ER-α:ER-β ratio correlated with ER status as assessed by ligand binding assay. Moreover, trends toward a positive correlation between ER-α and ER status and toward a negative correlation between ER-β and ER status were observed. Together, these data suggest that ligand binding is mainly due to the ER-α protein.

We have previously observed that the apparent ER-α:ER-β ratio in breast tumors varies widely (12). Our current results using the multiplex RT-PCR approach confirm and support these previous observations. Given the differential activity of tamoxifen-like antiestrogens through ER-α and ER-β, it is tempting to speculate that altered ratios of these receptors may be a possible mechanism associated with tamoxifen resistance.

In conclusion, our results provide evidence to support the hypothesis that altered ER-α and ER-β expression may have a significant role in alterations of estrogen action that occur during human breast cancer.

Acknowledgments

We thank M. Cheang (Medical Biostatistical Consulting Unit, Faculty of Medicine, University of Manitoba, Winnipeg, Canada) for help in analyzing data.

References

1. Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., and Chambon, P. Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature (Lond.), *320:* 134–139, 1986.
2. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R., and Chambon, P. Functional domains of the human estrogen receptor. Cell, *51:* 941–951, 1987.
3. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog. Horm. Res., *52:* 141–164, 1997.
4. Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J. Biol. Chem., *269:* 16433–16442, 1994.
5. Mosselman, S., Polman, J., and Dijkema, R. ER-β: identification and characterization of a novel human estrogen receptor. FEBS Lett., *392:* 49–53, 1996.
6. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. Cloning of a novel receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA, *93:* 5925–5930, 1996.
7. Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguere, V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol. Endocrinol., *11:* 353–365, 1997.
8. Pace, P., Taylor, J., Suntharalingam, S., Coombes, R. C., and Ali, S. Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. J. Biol. Chem., *272:* 25832–25838, 1997.
9. Watanabe, T., Inoue, S., Ogawa, S., Ishii, Y., Hiroi, H., Ikeda, K., Orimo, A., and Muramatsu, M. Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context, and estrogen receptor subtype: functional difference between estrogen receptors α and β. Biochem. Biophys. Res. Commun., *236:* 140–145, 1997.
10. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. Differential ligand activation of estrogen receptors ER-α and ER-β at AP1 sites. Science (Washington DC), *277:* 1508–1510, 1997.
11. Cowley, S. M., Hoare, S., Mosselman, S., and Parker, M. G. Estrogen receptors α and β form heterodimers on DNA. J. Biol. Chem., *272:* 19858–19862, 1997.
12. Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. Expression of estrogen receptor-β in human breast tumors. J. Clin. Endocrinol. Metab., *82:* 2371–2374, 1997.
13. Vladusic, E. A., Hornby, A. E., Guerra-Vladusic, F. K., and Lupu, R. Expression of estrogen receptor β messenger RNA variant in breast cancer. Cancer Res., *58:* 210–214, 1998.
14. Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J. A. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J. Clin. Endocrinol. Metab., *82:* 4258–4265, 1997.
15. Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., Murphy, L. C., and Watson, P. H. Estrogen receptor-β mRNA variants in human and murine tissues. Mol. Cell. Endocrinol., in press, 1998.
16. Henderson, B. E., Ross, R. K., Pike, M. C., and Casagrande, J. T. Endogenous hormones as a major factor in human cancer. Cancer Res., *42:* 3232–3239, 1982.
17. Hiller, T., Snell, L., and Watson, P. H. Microdissection RT-PCR analysis of gene expression in pathologically defined frozen tissue sections. Biotechniques, *21:* 38–44, 1996.
18. Khan, S. A., Rogers, M. A., Obando, J. A., and Tamsen, A. Estrogen receptor expression of benign breast epithelium and its association with breast cancer. Cancer Res., *54:* 993–997, 1994.
19. Jordan, V. C. Third annual William L. McGuire Memorial Lecture. Studies on the estrogen receptor in breast cancer: 20 years as a target for the treatment and prevention of cancer. Breast Cancer Res. Treat., *36:* 267–285, 1995.
20. Schmitt, F. C. Multistep progression from an oestrogen-dependent growth towards an autonomous growth in breast carcinogenesis. Eur. J. Cancer, *31:* 2049–2052, 1995.
21. Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E., and McInerney, E. M. William L. McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. Breast Cancer Res. Treat., *44:* 23–38, 1997.
22. Saric, T., and Shain, S. A. Semiquantitative RT-PCR: enhancement of assay accuracy and reproducibility. Biotechniques, *22:* 630–636, 1997.
23. Petersen, O. W., Hoyer, P. E., and van Deurs, B. Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. Cancer Res., *47:* 5748–5751, 1987.
24. Brandenberger, A. W., Tee, M. K., and Jaffe, R. B. Estrogen receptor α (ER-α) and β (ER-β) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-β in neoplastic tissues. J. Clin. Endocrinol. Metab., *83:* 1025–1028, 1998.

APPENDIX 4

British Journal of Cancer (1998) 00(0), 000–000

Oestrogen receptor-α variant mRNA expression in primary human breast tumours and matched lymph node metastases

E Leygue[1], RE Hall[2], H Dotzlaw[1], PH Watson[3] and LC Murphy[1]

[1]Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, MB, Canada; [2]Department of Surgery, Flinders University of South Australia, Bedford Park, SA, Australia; [3]Department of Pathology, University of Manitoba, Winnipeg, MB, Canada

Summary We have shown previously that the relative expression of a truncated oestrogen receptor-α variant mRNA (ER clone 4) is significantly increased in axillary node-positive primary breast tumours compared with node-negative tumours. In this study, we have examined the relative expression of clone 4-truncated, exon 5-deleted and exon 7-deleted oestrogen receptor-α variant mRNAs in 15 primary breast tumour samples and in synchronous axillary lymph node metastases. Overall, there were no significant differences between the primary tumours and the matched metastases in the relative expression of these three specific variant mRNAs. Furthermore, the pattern of all deleted oestrogen receptor-α variant mRNAs appeared conserved between any primary and its matched secondary tumour.

Keywords: oestrogen receptor-α variants; breast cancer; metastasis

Multiple oestrogen receptor-α (ER) mRNA species have been identified in human breast cancer samples (Dowsett et al, 1997; Murphy et al, 1997*a*, *b*). The significance of these variant transcripts remains unclear. Although the ability to detect variant ER proteins encoded by such variant transcripts remains controversial (Park et al, 1996; Desai et al, 1997; Huang et al, 1997), alteration of expression of some variant ER mRNAs has been found to occur during both breast tumorigenesis (Leygue et al, 1996*a*, *b*) and breast cancer progression. With regard to the latter, we showed previously that the expression of the truncated, clone 4 variant (C4) ER mRNA (Dotzlaw et al, 1992) was significantly increased relative to wild-type (WT) ER mRNA in a group of primary breast tumours with multiple poor prognostic features compared with a group of primary breast tumours with good prognostic features (Murphy et al, 1995). The 'poor' prognostic features were defined as the presence of lymph node metastases at the time of surgery, large tumour size, lack of progesterone receptor (PR) expression and high S-phase fraction, while 'good' prognostic features were lack of nodal involvement, small tumour size, PR positivity and low S-phase fraction. In the same study, the relative expression of clone 4 ER variant mRNA was significantly higher in primary breast tumours that were PR– than in those that were PR+ (Murphy et al, 1995). This suggested that altered ER variant expression may be a marker of a more aggressive phenotype and lack of endocrine sensitivity in human breast cancer. As a prerequisite to addressing such a possibility, we have investigated the pattern of ER variant expression in a cohort of primary tumours and their matched, concurrent lymph node metastases.

Received 20 April 1998
Revised 3 July 1998
Accepted 14 July 1998

Correspondence to: LC Murphy

MATERIALS AND METHODS

Tumour selection and RNA isolation

Sections from 15 frozen primary human breast tumour samples and their matched frozen lymph node metastases were provided by the Manitoba Breast Tumour Bank (Winnipeg, MB, Canada). For the primary tumour samples, the ER levels, determined by ligand-binding assays, ranged from 0.8 fmol mg^{-1} protein to 89 fmol mg^{-1} protein with a median value of 17.5 fmol mg^{-1} protein. Thirteen tumours were ER+ and two were ER– (ER+ was defined as >3 fmol mg^{-1} protein). PR levels determined by ligand-binding assays ranged from 2.9 fmol mg^{-1} protein to 112 fmol mg^{-1} protein with a median value of 12.6 fmol mg^{-1} protein. Nine tumours were PR+ and 6 were PR– (PR+ was defined as > 10 fmol mg^{-1} protein). ER and PR values were available for only four of the lymph node metastases and the ER and PR status as defined by ligand binding did not differ from their matched primary tumour. RNA was extracted from the sections using Trizol reagent (Gibco/BRL, Ontario, Canada) according to the manufacturer's instructions.

For validation of triple-primer polymerase chain reactions (TP-PCR) by comparison with RNAase protection assays, a second cohort of human breast tumour specimens (25 cases) was also obtained from the Manitoba Breast Tumour Bank. Twenty of these tumours were ER+, as determined by ligand-binding assay, with values ranging from 4.5 to 311 fmol mg^{-1} protein (median 93 fmol mg^{-1}). The five remaining cases were ER–, with values ranging from 0 to 1.8 fmol mg^{-1} protein (median 0.9 fmol mg^{-1}). Total RNA was extracted from frozen tissues using guanidinium

thiocyanate as previously described (Murphy and Dotzlaw, 1989). The integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (Murphy and Dotzlaw, 1989).

RNase protection assay

Antisense riboprobes spanning the point at which the C4 ER mRNA sequence diverges from the WT ER mRNA sequence (Dotzlaw et al, 1992) were synthesized as previously described (Dotzlaw et al, 1990). The level of C4 ER mRNA and WT ER mRNA in 10 µg of total RNA was determined using an RNAase Protection Assay kit (RPA II, Ambion, Austin, TX, USA) following the manufacturer's instructions. Briefly, RNA was denatured at 80°C for 5 min in the presence of 5×10^5 d.p.m. ^{32}P-labelled riboprobe, then hybridized at 42°C for 16 h. Following RNAase digestion, samples were electrophoresed on 6% acrylamide gels containing 7 M urea, dried and autoradiographed.

To quantify C4 and WT ER mRNAs within breast tumour samples, a standard curve was established in each assay. C4 and WT ER mRNAs (30, 125, 500 pg C4 RNA and 125, 500, 2000 pg WT ER RNA) synthesized using T7 RNA polymerase were purified on a Sephadex G-50 column and quantitated spectrophotometrically. WT ER RNA was transcribed from linearized pHEO, which contains the entire WT ER coding sequence but is missing the 3′-untranslated portion of the ER mRNA [(kindly provided by P Chambon, Strasbourg, France (Green et al, 1986)]. Full-length C4 RNA was transcribed from linearized pSK-C4 (Dotzlaw et al, 1992). Standard RNAs were analysed together in the same assay as the breast tumour mRNAs. Bands corresponding to the C4 ER mRNA and WT ER mRNA protected fragments were excised from the gel and counted after addition of 5 ml scintillant (ICN Pharmaceuticals, Inc., Irvine, CA, USA) in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA). For each sample, absolute amounts of C4 and WT ER mRNA were determined from the standard curve.

Reverse transcription, PCR and triple-primer (TP) PCR

For each sample, 1 µg of total RNA was reverse transcribed in a final volume of 15 µl as described previously (Leygue et al, 1996*a*). One microlitre of the reaction mixture was taken for subsequent amplification.

The primers and PCR conditions for the long-range PCR were as previously described (Leygue et al, 1996*c*). The primers and PCR conditions for measuring the relative expression of exon 5-deleted and exon 7-deleted ER transcripts relative to WT ER transcripts were as previously described (Leygue et al, 1996*a*).

The TP-PCR conditions were similar to those previously described (Leygue et al, 1996*b*) with minor modifications. ERU (5′-TGTGCAATGACTATGCTTCA-3′, sense, located in WT ER exon 2; 792–811, as numbered in Green et al, 1986) and ERL (5′-GCTCTTCCTCCTGTTTTTAT-3′, antisense, located in WT ER exon 3; 921–940) primers allowed amplification of a 148-bp fragment corresponding to WT ER mRNA. The C4-specific primer (C4L, 5′-TTTCAGTCTTCAGATACCCCAG-3′, antisense; 1315–1336, as numbered in Dotzlaw et al, 1992) spans the only region of the C4 unique sequence that does not have any homology with repetitive LINE-1 sequences (Dotzlaw et al, 1992). ERU and C4L allowed amplification of a 536-bp fragment corresponding specifically to C4 ER variant mRNA.

PCR amplifications were performed in a final volume of 10 µl in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM potassium chloride, 2 mM magnesium chloride, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng µl^{-1} of each primer (ERU, ERL and C4L), 0.2 units of *Taq* DNA polymerase (Gibco-BRL) and 1 µCi of [α-^{32}P] dCTP (3000 Ci mmol^{-1}, ICN Pharmaceuticals, Irvine, CA, USA). Each PCR consisted of 30 cycles (1 min at 94°C, 30 s at 60°C and

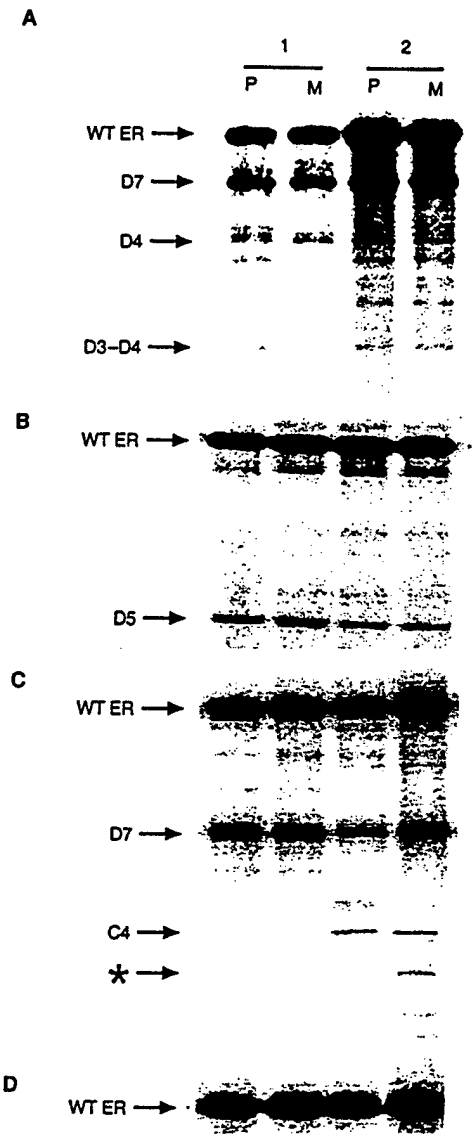


Figure 1 (A) Autoradiogram of long-range RT-PCR (Leygue et al, 1996*c*) results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastasis (M). WT ER is the expected product corresponding to the WT ER mRNA; D7 is the expected product corresponding to the exon 7-deleted ER variant mRNA; D4 is the expected product for the exon 4-deleted ER mRNA; D3-4 is the expected product for the exon 3+4-deleted ER mRNA; D4/7 is the expected product for the exon 4+7-deleted ER mRNA. (B) Autoradiogram of RT-PCR results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastasis (M). D5 is the expected product corresponding to the exon 5-deleted ER variant mRNA. WT ER is the expected product corresponding to the WT ER mRNA. (C) Autoradiogram of RT-PCR results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastasis (M). D7 is the expected product corresponding to the exon 7-deleted ER variant mRNA. WT ER is the expected product corresponding to the WT ER mRNA. (D) Autoradiogram of TP-PCR results from two samples of primary breast tumours (P) and their matched concurrent lymph node metastasis (M). C4 is the expected product corresponding to the clone 4 ER variant mRNA. WT ER is the expected product corresponding to the WT ER mRNA. *Band coamplified with C4 and WT ER and shown to correspond to an exon 2-duplicated ER variant mRNA

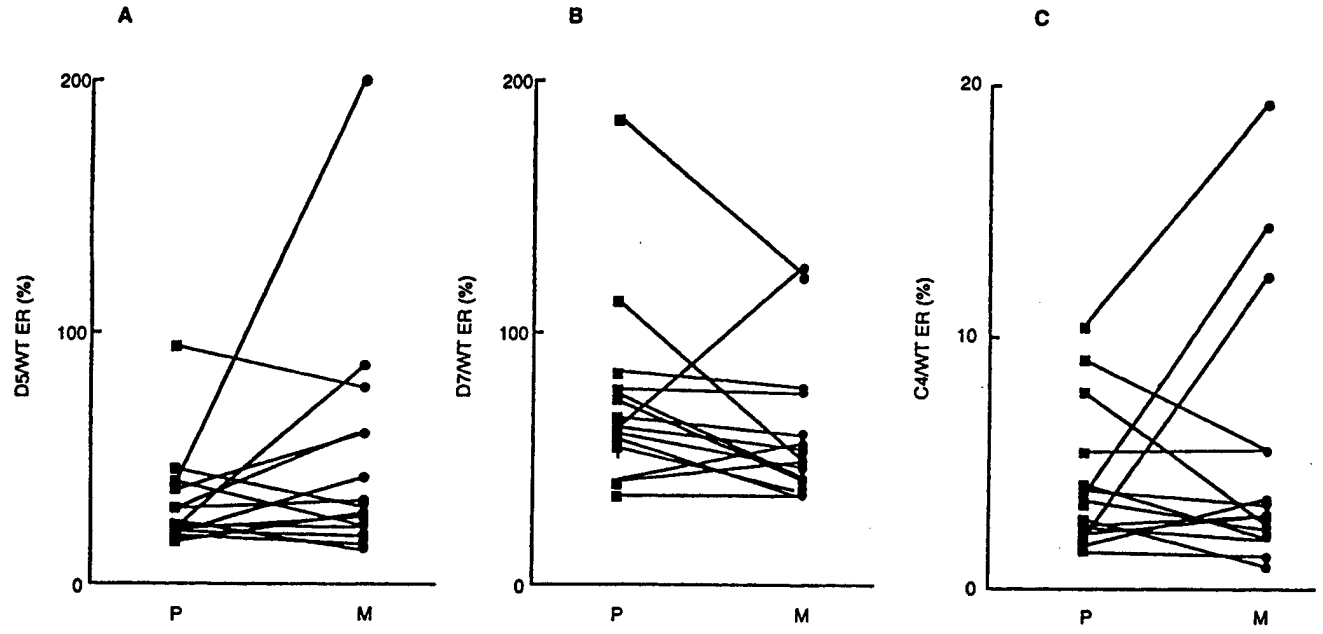


Figure 2 (A) Quantitative comparison of the relative expression of exon 5-deleted variant ER mRNA in primary (P) human breast tumours and their concurrent matched lymph node metastases (M). For each sample, the mean of three independent measures of exon 5-deleted ER relative expression expressed as a percentage of the corresponding WT ER signal was determined as described in the Materials and methods section. (B) Quantitative comparison of the relative expression of exon 7-deleted variant ER mRNA in primary (P) human breast tumours and their concurrent matched lymph node metastases (M). For each sample, the mean of three independent measures of exon 7-deleted ER relative expression expressed as a percentage of the corresponding WT ER signal was determined as described in the Materials and methods section. (C) Quantitative comparison of the relative expression of clone 4 variant ER mRNA in primary (P) human breast tumours and their concurrent matched lymph node metastases (M). For each sample, the mean of three independent measures of clone 4 relative expression expressed as a percentage of the corresponding WT ER signal was determined as described in the Materials and methods section

1 min at 72°C) using a thermocycler (Perkin Elmer). Four microlitres of the reaction mix was then denatured by addition of 6 µl of 80% formamide buffer and boiling before electrophoresis on 6% polyacrylamide gels containing 7 M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at –70°C with two intensifying screens for 2 h.

Quantification of RT-PCR and TP-PCR

Bands corresponding to the variant ER mRNA and WT ER mRNA were excised from the gel and counted after addition of 5 ml of scintillant in a scintillation counter. The variant signal was expressed as a percentage of the WT ER signal. It should be noted that the percentage obtained reflects the relative ratio of the variant to WT ER RT-PCR product and does not provide absolute initial mRNA levels. Validation of this approach was described previously (Daffada et al, 1994, 1995; Leygue et al, 1996a,b). At least two independent PCR assays were performed for each sample in the comparison of RNAase protection assay with TP-PCR assays. For assessment of matched primary and secondary breast tumour samples, at least two and in most cases three independent PCR reactions were performed and the mean determined.

The statistical significance of differences in the relative levels of expression of any single ER mRNA variant between primary tumour and lymph node metastasis was determined using the Wilcoxon signed-rank test.

RESULTS

Determination of the pattern of exon-deleted ER variant mRNA expression

Multiple ER variant mRNAs have been shown to be expressed in any one breast tissue sample (Leygue et al, 1996a; Murphy et al, 1997a, b). To investigate the pattern of multiple exon-deleted ER variant expression between primary breast tumours and their matched lymph node metastases, a long-range RT-PCR approach was used. This approach, based on the competitive amplification of wild-type and exon-deleted ER variant cDNAs, using primers annealing within exons 1 and 8, allows the evaluation of the relative pattern of expression of all exon-deleted ER variant transcripts present in any individual sample (Leygue et al, 1996c; Fasco, 1997). Typical results are shown in Figure 1A. The pattern of deleted ER mRNA expression between any one primary tumour and its matched lymph node metastasis was conserved.

Determination of the relative expression of exon 5-deleted and exon 7-deleted ER variant mRNA expression

Using a previously validated semiquantitative PCR approach (Leygue et al, 1996a), the measurement of the relative expression of specific individual exon-deleted ER variant mRNAs was also undertaken. Specifically, the relative expressions of exon 5-deleted

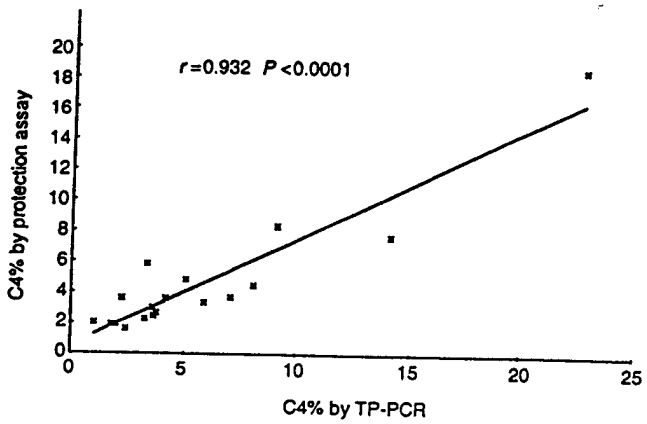


Figure 3 Linear regression analysis of clone 4 expression (expressed as a percentage of the corresponding WT ER expression) as determined by TP-PCR vs standardized RNAase protection assay in 18 human breast tumours

ER cDNA (Figure 1B) using primers in exons 4 and 6, and exon 7-deleted ER cDNA (Figure 1C), using primers in exons 5 and 8, were measured. The median value for the relative expression of the exon 5-deleted ER for the primary tumours was 23.1% (range 17.3–94.3%) and the median value for the matched lymph node metastases was 31.3% (range 14.9–200%). The scatter plot for these results is shown in Figure 2A. The median relative expression of the exon 7-deleted ER for primary tumours was 65% (range 39.3–184.9%) and the median value for the matched lymph node metastases was 52.5% (range 35.5–126%). The scatterplot of these results is shown in Figure 2B. There were no statistically significant differences in the relative expression of either exon-deleted ER mRNA between primary and concurrent metastatic tumours.

Comparison of RNAase protection assay and triple-primer PCR assay for determination of the relative expression of clone 4 truncated ER variant mRNA expression

Another frequently expressed ER variant, which would not be detected in the above assays, is the C4 ER mRNA. This variant was previously found to be significantly elevated in a group of primary breast tumours with poor prognostic features that included concurrent lymph node metastases, compared with a group of primary tumours with good prognostic variables that included lack of concurrent nodal metastases (Murphy et al, 1995). Therefore, it was relevant to determine the level of C4 ER variant expression in primary breast tumours and their matched, concurrent lymph node metastases.

In this previous study, we used RNAase protection assays to measure WT and variant ER mRNA expression (Murphy et al, 1995). However, in order to conduct this study using smaller tissue samples (in particular from nodal metastases) and to ensure a close correlation with the histological composition of the tissue, we used a previously described TP-PCR assay (Leygue et al, 1996*b*) to measure the relative expression of C4 ER mRNA. To facilitate comparison of the current data with our earlier study (Murphy et al, 1995), it was necessary to compare the RNAase protection assay with the TP-PCR assay, before proceeding to analyse the primary and secondary breast tumour samples for C4 mRNA expression by TP-PCR.

RNA from 25 human breast tumours, selected to represent a wide range of ER status by ligand-binding assay (Table 1), was

Table 1 C4 and WT ER mRNA expression in 25 human breast tumours, as determined by RNAase protection assay and TP-PCR

Sample no.	Ligand binding	RNAase protection			TPPCR
	ER (fmol mg^{-1})	C4 (pg 10 μg^{-1})	WT ER (pg 10 μg^{-1})	C4 (%)	C4 (%)
5	0.0	ND	ND	–	1.7
3	0.4	ND	ND	–	2.6
1	0.9	ND	ND	–	3.1
24	1.2	6.2	105.1	5.9	3.3
4	1.8	ND	ND	–	3.7
23	4.5	10.0	54.3	18.4	22.7
8	5.8	ND	26.8	–	2.8
7	6.3	ND	224.6	–	3.4
2	8.7	ND	9.0	–	2.2
19	10.0	22.6	902.9	2.5	3.6
10	17.8	5.3	146.4	3.6	4.1
13	25.0	2.3	112.0	2.0	1.0
15	44.0	5.0	148.5	3.4	5.9
22	57.0	11.8	153.6	7.7	14.1
11	90.0	2.5	129.1	1.9	1.7
21	96.0	9.6	263.4	3.6	2.2
14	105.0	4.6	94.4	4.9	5.0
17	111.0	26.7	320.3	8.3	9.1
9	121.0	4.6	277.7	1.7	2.4
6	146.0	2.0	105.0	1.9	1.9
18	198.0	15.8	422.0	3.7	7.0
20	236.0	8.8	288.4	3.0	3.5
12	289.0	3.6	80.5	4.5	8.0
16	304.0	38.8	1440.8	2.7	3.7
25	311.0	83.9	3651.0	2.3	3.2

ND, not detected.

analysed in a standardized RNAase protection assay in order to determine the absolute amount of C4 and WT ER mRNAs within each sample. The signals corresponding to C4 and WT ER mRNAs were quantified as described in Materials and methods. In each assay, known amounts of synthetic WT ER and C4 mRNAs were analysed in parallel in order to establish a standard curve allowing the determination of absolute levels of C4 and WT ER mRNAs, expressed as pg 10 μg^{-1} RNA (Table 1). Because of the very low C4 protected fragment signal (≤ 15 d.p.m.) in seven tumours, it was not possible to determine confidently the absolute amount of C4 mRNA in these samples (not determined, ND). All C4-negative tumours by RNAase protection assay were from tumours with ER values lower than 10 fmol mg^{-1} protein, as determined by ligand-binding assay. The absolute amounts of C4 and WT ER mRNAs in the remaining 18 tumours, as determined by RNAase protection assay, varied from 2 to 83.9 pg 10 μg^{-1} RNA and from 9 to 3651 pg 10 μg^{-1} RNA respectively. For each sample, the C4 mRNA signal was expressed as a percentage of WT ER mRNA signal (Table 1).

C4 ER mRNA relative expression was determined by TP-PCR within the same 25 RNA samples as described in Materials and methods. Both C4 and WT ER cDNAs signals were detected in all 25 tumours studied, independent of their ER status as determined by ligand-binding assay. C4 and WT ER signals were quantified as described in Materials and methods. The signal corresponding to C4 was expressed as a percentage of the WT ER signal. Table 1 presents the average of a least two independent TP-PCR experiments. Linear regression analysis (Figure 3) shows a highly significant correlation between C4 mRNA relative expression as

determined by RNAase protection assay (in the 18 tumours in which a C4 signal was detectable) and C4 mRNA relative expression determined by TP-PCR ($r = 0.932$, $P < 0.0001$). Interestingly, an additional band was also observed in most of the samples using the TP-PCR assay (see asterisk, Figure 1D). This band was identified after subcloning and sequencing to be a product of an exon 2-duplicated ER variant mRNA. The intensity of the signal obtained from this exon 2-duplicated ER band paralleled that of the WT ER band, and the co-amplification of the exon 2-duplicated ER variant mRNA using TP-PCR did not interfere with the relationship between TP-PCR and RNAase protection assay.

Determination of the relative expression of clone 4 truncated ER variant mRNA expression

The above TP-PCR assay was used to compare the relative expression of C4 and WT ER expression in the matched breast cancer samples (Figure 1D). The median relative expression of the C4 ER for the primary tumours was 3.5% (range 1.6–10.5%) and the median value for the matched lymph node metastases was 3.1% (range 1.0–19.4%). A scatterplot of the results is shown in Figure 2C. There is no statistically significant difference in the relative expression of C4 ER variant expression between primary breast tumours and their concurrent lymph node metastases by Wilcoxon rank-sum analysis. Interestingly, although not statistically significant, we found that the median level of C4 expression in ER+ PR– primary tumours, 3.7% (range 2.5–7.9%, $n = 5$), was approximately 50% higher than the median level of C4 expression in ER+ PR+ primary tumours, which was 2.4% (range 1.6–10.5%, $n = 8$). Such a trend would be consistent with our previous results in which C4 expression was higher in PR– primary breast tumours than in PR+ primary tumours.

DISCUSSION

The data presented in this study provide evidence that both the overall pattern of ER variant expression and the relative level of expression of three individual ER variants are conserved in primary breast tumours and their matched, concurrent lymph node metastases.

The observations presented in this manuscript, showing a conserved pattern and similar relative expression of ER variants between primary tumours and their concurrent lymph node metastases, would be consistent with previous observations that little change of ER status can be found between primary human breast tumours and their concurrent lymph node metastases or their distant metastases (Hahnel and Twaddle, 1985; Robertson, 1996). These findings are not inconsistent with our previously published data, which showed that the relative expression of one ER variant was significantly increased in primary tumours with poor prognostic characteristics, which included having concurrent lymph node metastases, as compared with primary tumours without concurrent lymph node metastases (Murphy et al, 1995). It should be stressed that all the primary tumours in the current study had concurrent lymph node metastases, a major feature of poor prognosis in breast cancer, and most likely resembled our previously described poor prognostic group (Murphy et al, 1995). Therefore in primary tumours that have concurrent lymph node metastases and have detectable levels of C4 ER variant as well as other variant ER mRNAs, mRNA levels do not significantly change between primary tumours and their concurrent lymph node metastases. These data do not, however, shed any light on whether tumours with good prognostic features, as previously described (Murphy et al, 1995), that have a relatively low level of C4 ER variant mRNA subsequently develop higher levels when recurrent disease develops. Although this issue remains to be investigated, our earlier observation of higher relative C4 ER mRNA expression in PR– primary tumours compared with PR+ primary tumours appeared to be conserved in the present cohort, although the numbers were low and the difference did not reach statistical significance. As quantitative differences in the expression of several ER variants have been shown to occur in primary breast tumours compared with normal human breast tissues (Leygue et al, 1996a,b), as well as between good vs poor prognosis primary breast tumours, the current data suggest that alterations in ER variant expression and any role this may have in altered oestrogen signal transduction probably occurs early in tumorigenesis and well before the acquisition of the ability to metastasize. This is consistent with previous data supporting the concept of an early involvement of perturbations of oestrogen signal transduction and the development of hormone independence in breast tumorigenesis (Khan et al, 1994; Schmitt, 1995). It remains therefore to be determined if altered ER variant expression can predict tumour recurrence and progression in node-negative breast cancers.

To our knowledge, this study is the first to compare an already established quantitative approach, such as the RNAase protection assay, with an RT-PCR based approach in the study of ER variant mRNA expression. Earlier studies have utilized either the RNAase protection assay ~~only~~ or ~~by~~ RT-PCR only. Considering the potential clinical relevance of the measurement of the relative level of ER variants with respect to WT ER within human breast tissue samples and the sensitivity of an RT-PCR based approach, such a comparative study was deemed necessary. Furthermore, our data provide validation for comparing previous data obtained using a non-amplification-dependent RNAase protection assay with the current data obtained using an amplification-dependent TP-PCR assay.

The lack of sensitivity of the RNAase protection assay for a subset of tumours with very low (<10 fmol mg^{-1}) ER values by ligand-binding assay is an important limiting factor. It effectively means that, in a screening study, ER-negative tumours (<3 fmol mg^{-1} protein), as well as ER-positive tumours with ER values lower than 10 fmol mg^{-1}, as measured by ligand-binding assay, cannot be reliably assessed for C4 ER variant mRNA expression by RNAase protection assay. This, together with the relatively large amount of RNA needed to perform an RNAase protection analysis, severely limits the usefulness of a standardized RNAase protection assay in such screening studies. The low amount of starting material needed, together with the higher sensitivity observed (samples C4 ER variant negative by RNAase protection assay had detectable levels of C4 ER variant and WT ER mRNA by TP-PCR) make TP-PCR an attractive alternative to the RNAase protection assay in studies in which such factors are limiting.

In conclusion, the current investigation extends our previous studies on the relationship of ER variant expression and progression in human breast cancer. The data presented show that both the pattern and level of expression of ER variants are conserved between matched primary breast tumours and their concurrent lymph node metastases. Therefore, any alteration in ER variant expression that could be a marker of altered ER signal transduction and breast cancer progression probably occurs before breast cancer cells acquire the ability to metastasize.

ACKNOWLEDGEMENTS

This work was supported by the US Army Medical Research and Materiel Command (USAMRMC), grant number DAMD17-95-1-5015. The Manitoba Breast Tumour Bank is supported by funding from the National Cancer Institute of Canada (NCIC). LCM is a Medical Research Council of Canada (MRC) Scientist, PHW is a MRC Clinician-Scientist, EL is a recipient of a USAMRMC Postdoctoral Fellowship (DAMD17-96-1-6174), RH was supported by a University of Manitoba Visiting Scientist Award.

REFERENCES

Daffada AA, Johnston SR, Nicholls J and Dowsett M (1994) Detection of wild type and exon 5-deleted splice variant oestrogen receptor (ER) mRNA in ER-positive and -negative breast cancer cell lines by reverse transcription/polymerase chain reaction. *J Mol Endocrinol* 13: 265–273

Daffada AA, Johnston SR, Smith IE, Detre S, King N and Dowsett M (1995) Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. *Cancer Res* 55: 288–293

Desai A, Luqmani Y, Coope R, Dagg B, Gomm J, Pace P, Rees C, Thirunavukkarasu V, Shousha S, Groome N, Coombes R and Ali S (1997) Presence of exon 5 deleted oestrogen receptor in human breast cancer: functional analysis and clinical significance. *Br J Cancer* 75: 1173–1184

Dotzlaw H, Miller T, Karvelas J and Murphy LC (1990) Epidermal growth factor gene expression in human breast cancer biopsy samples: relationship to estrogen and progesterone receptor gene expression. *Cancer Res* 50: 4204–4208

Dotzlaw H, Alkhalaf M and Murphy LC (1992) Characterization of estrogen receptor variant mRNAs from human breast cancers. *Mol Endocrinol* 6: 773–785

Dowsett M, Daffada A, Chan C and Johnston S (1997) Oestrogen receptor mutants and variants in breast cancer. *Eur J Cancer* 33: 1177–1183

Fasco M (1997) Quantitation of estrogen receptor mRNA and its alternatively spliced mRNAs in breast tumor cells and tissues. *Anal Biochem* 245: 167–178

Green S, Walter P, Kumar V, Krust A, Bornert J, Argos P and Chambon P (1986) Human estrogen receptor cDNA: sequence, expression, and homology to v-erb-A. *Nature* 320: 134–139

Hahnel R and Twaddle E (1985) The relationship between estrogen receptors in primary and secondary breast carcinomas and in sequential primary breast carcinomas. *Breast Cancer Res Treat* 5: 155–163

Huang A, Leygue E, Snell L, Murphy LC and Watson P (1997) Expression of estrogen receptor variants mRNAs and determination of estrogen status in human breast cancer. *Am J Pathol* 150: 1827–1833

Khan SA, Rogers MA, Obando JA and Tamsen A (1994) Estrogen receptor expression of benign breast epithelium and its association with breast cancer. *Cancer Res* 54: 993–997

Leygue ER, Watson PH and Murphy LC (1996*a*) Estrogen receptor variants in normal human mammary tissue. *J Natl Cancer Inst* 88: 284–290

Leygue E, Murphy LC, Kuttenn F and Watson P (1996*b*) Triple primer polymerase chain reaction. A new way to quantify truncated mRNA expression. *Am J Pathol* 148: 1097–1103

Leygue E, Huang A, Murphy LC and Watson P (1996*c*) Prevalence of estrogen receptor variant messenger RNAs in human breast cancer. *Cancer Res* 56: 4324–4327

Murphy LC and Dotzlaw H (1989) Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. *Mol Endocrinol* 3: 687–693

Murphy LC, Hilsenbeck SG, Dotzlaw H and Fuqua SAW (1995) Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. *Clin Cancer Res* 1: 155–159

Murphy LC, Dotzlaw H, Leygue E, Douglas D, Coutts A and Watson P (1997*a*). Estrogen receptor variants and mutations: a review. *J Steroid Biochem Mol Biol* 62: 363–372

Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A and Watson P (1997*b*) Oestrogen receptor variants and mutations in human breast cancer. *Ann Med* 29: 221–234

Park W, Choi J, Hwang E and Lee J (1996) Identification of a variant estrogen receptor lacking exon 4 and its coexpression with wild type estrogen receptor in ovarian carcinomas. *Clin Cancer Res* 2: 2029–2035

Robertson J (1996) Oestrogen receptor: a stable phenotype in breast cancer. *Br J Cancer* 73: 5–12

Schmitt F (1995) Multistep progression from an oestrogen dependent growth towards an autonomous growth in breast carcinogenesis. *Eur J Cancer* 12: 2049–2052

APPENDIX 5

Variant estrogen receptor-alpha messenger RNA expression in hormone-independent human breast cancer cells.

A S Coutts, E Leygue and L C Murphy*.

Department of Biochemistry and Molecular Biology, University of Manitoba, 770 Bannatyne Ave. Winnipeg, Manitoba, Canada R3E 0W3.

* To whom correspondence is addressed

Telephone: (204) 789 3233

Fax: (204) 789 3900

Email: lcmurph@cc.umanitoba.ca

Running title: Variant estrogen-receptor alpha and estrogen-independence.
Key words: Estrogen receptor, estrogen-independence, variant, breast cancer, exon deletion.

Abstract

The development of estrogen-independent growth is thought to be an important step in the progression of breast cancer to hormone-independence and endocrine therapy resistance. T5 human breast cancer cells are estrogen receptor (ER-α) positive and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells, by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen while still retaining expression of the ER-α. In the apparent absence of ligand T5-PRF cells have a 3.6 ± 0.5 fold increased basal ER-α transcriptional activity and elevated basal progesterone receptor (PR) levels compared to the parent T5 cells. Long range ER-α reverse transcription-polymerase chain reaction (RT-PCR) was performed to characterize the pattern of variant ER–α mRNA expression between the two cell lines and a differential expression of an ER-α mRNA variant was found. In particular, an ER–α variant mRNA, deleted in exons 3 and 4, was detected only in T5-PRF cells. Recombinant expression of this ER–α variant confered increased basal transcriptional activity and estrogen-responsiveness when expressed with wild-type ER-α in ER negative cell lines, as well as increasing both ligand-independent and estrogen-induced ER-α transcriptional activity when expressed alone in parental T5 cells. These results suggest a possible role for the altered expression of an ER-α variant in ligand-independent activation of ER-α which may contribute to the estrogen-independent phenotype in T5-PRF human breast cancer cells.

Introduction

Breast cancer is a hormonally responsive cancer and hormones, including estrogen, are required for breast cancer growth (1). Estrogens promote the growth of human breast cancer, and as such, most endocrine therapies are aimed at blocking the growth promoting effects of estrogen (e.g., antiestrogen such as tamoxifen). Breast cancers are classified according to their requirement for proliferation as being either hormone-dependent or hormone-independent, based ultimately on the response to endocrine therapy of metastatic disease (2). The level of estrogen receptor-alpha (ER-α) in human breast cancer (HBC) is used as a marker not only of potential therapeutic response to endocrine therapy, but is a marker of prognosis and survival (3).

The evolution of breast cancer into an estrogen-independent growth phenotype is thought to be an important step in the progression of breast cancer to hormone-independence and endocrine therapy resistance (4, 5). Understanding the factors that contribute to the development of a hormone-independent phenotype is of major importance in terms of breast cancer therapeutics. Resistance to endocrine therapies may be due to a number of factors. In some cases, hormone-independence and resistance can occur due to loss of ER expression, but most tumours that have developed resistance to endocrine therapy remain receptor positive (6).

Several breast cancer cell lines in culture also require estrogen for growth and long-term culture in estrogen-depleted conditions can result in cells becoming apparently independent of the requirement for estrogen for growth. Indeed, the development of estrogen-independent growth in human breast cancer is thought to be one of the initial steps in the progression to hormone-independence and resistance to endocrine therapies (7). However, the mechanisms responsible for the development of estrogen-independence in the presence of continued expression of ER-α are poorly understood. In order to address this we have developed a breast cancer cell model of apparent estrogen independence (8). T5 human breast cancer cells are ER-α positive and estrogen treatment in culture results in

increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line while still retaining expression of the ER-α (8). However, these cells remain sensitive to the growth inhibitory effects of 4-hydroxy-tamoxifen (OT) and ICI 164,384 (ICI), although they have reduced sensitivity to ICI compared to the parent T5 cells (8).

In this study we have investigated the ligand-dependent and -independent transcriptional activity of the endogenous ER-α as well as the pattern and potential function of ER-α variant expression in T5 and T5-PRF human breast cancer cells.

Materials and Methods

Materials

[^{32}P]dCTP and [^{35}S]ATP were purchased from ICN (St-Laurent, Quebec). Dulbecco's Minimal Essential Medium (DMEM) powder and fetal bovine serum were purchased from GIBCO/BRL (Burlington, Ontario). Horse serum and EGF were purchased from UBI (Lake Placid, New York). All other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario). Cholera toxin, 4-hydroxy-tamoxifen, estradiol-17β and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). [^{14}C]-chloramphenicol, [^{3}H]- R5020 (88.7 Ci/mmol), [^{35}S]-methionine and R5020 were obtained from NEN (Lachire, Quebec). ICI 164,384 was a gift from ICI (Macclesfield, Cheshire).

Cells and Cell Culture

T5 cells, previously called T-47D5, were originally thought to be a T-47D subline, however, DNA fingerprinting analysis showed that they were an MCF-7 subline (9). T5 and MDA-MB-231 human breast cancer cells were routinely cultured in DMEM containing 5% vol/vol fetal calf serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin. T5-PRF cells were routinely cultured in phenol red-free DMEM supplemented with 5% vol/vol twice charcoal dextran stripped fetal calf serum and 1% wt/vol glucose, glutamine, and penicillin-streptomycin (PRF/DMEM). MCF10A1 human breast epithelial cells (10) were grown routinely in DMEM containing 5% vol/vol horse serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin, 0.1μg/ml cholera toxin, 20ng/ml hEGF, 10.4μg/ml bovine insulin and 1μM hydrocortisone (DMEM-special). Transient transfections and steroid receptor assays were performed in PRF/DMEM. Transient transfections using MCF10A1 cells were performed in phenol red-free DMEM containing 5% vol/vol charcoal-stripped horse serum, 1% wt/vol glucose, glutamine and penicillin-streptomycin (PRF/DMEM-hs) and cells were passaged once prior to transfection in phenol red-free DMEM containing 5% vol/vol charcoal-stripped horse serum, 1% wt/vol glucose, glutamine, penicillin-streptomycin, 0.1μg cholera toxin, 20ng/ml hEGF, 10.4μg/ml bovine insulin and 1μM hydrocortisone (PRF/DMEM-special).

Progesterone Receptor Assays

PR assays were performed using whole cell ligand binding assays as previously described (11). [^{3}H]-R5020 and [^{3}H]-R5020 and 100 fold molar excess unlabelled R5020 were used to determine PR total and nonspecific binding, respectively. All assays were performed in the presence of 100 nM dexamethasone to prevent binding of R5020 to the glucocorticoid receptor.

Transient transfections and CAT assays

T5, T5-PRF and MDA-MB-231 cells were passaged once in PRF/DMEM and set up in 100 mm diameter dishes at 0.5 X 10^6 cells per dish in PRF/DMEM the day before transfection. MCF10A1 cells were passaged once in PRF/DMEM-special and set up in 100 mm diameter dishes at 2 X 10^6 cells per dish in PRF/DMEM-special two days before transfection. The following day the medium was changed to PRF/DMEM-hs and cells were transfected the following day, using the calcium phosphate/glycerol shock method (12) using an equal volume 2 x BBS buffer (50mM BES, 280mM NaCl, 1.5mM Na_2HPO_4 pH 6.95), followed by a 2 minute glycerol shock (20% vol/vol). Cells were washed twice with PBS and given fresh medium plus or minus 10 nM estradiol-17β (E2), 10nM estradiol-17β plus 1μM ICI 164,384 or 1μM ICI 164,384 alone. After 24h of treatment, the cells were harvested, cell extracts prepared and chloramphenicol acetyltransferase (CAT) activity measured (13). Transfection efficiency was determined by cotransfection of pCH110 (β-galactosidase expression vector, Pharmacia) and assay of β-galactosidase activity (14). T5 and T5-PRF cells were transfected with 5μg of ERE-tk-CAT (15), to determine ER–α transcriptional activity, along with 5μg pCH110. In the experiments where activity of d3/4 was examined in T5 cells, transfections were performed using 5μg ERE-tk-CAT, 5μg pCH110 plus or minus d3/4 expression vector (0.1-1 pmol) or vector DNA alone. MDA-MB-231 and MCF10A1 cells were transfected with 5μg ERE-tkCAT, 5μg pCH110, plus or minus 0.5pmol HEGO (wild-type ER-α expression vector, kindly provided by Dr P.

Chambon) with increasing amounts (0.5-2 pmol) of d3/4 expression vector or vector DNA alone.

Long-Range ER-α RT-PCR

Total RNA was extracted (Trizol reagent, GibcoBRL, Grand Island, NY) and reverse transcribed as described previously (16). The primer pair used consisted of 1/8U primer (5'-TGCCCTACTACCTGGAGAACG-3', sense; located in WT-ER-α exon 1; nucleotides 615-637) and 1/8L primer (5'-GCCTCCCCCGTGATGTAA-3', antisense; located in WT-ER-α exon 8; nucleotides 1995-1978). Nucleotide positions given correspond to published sequences of the human ER-α cDNA (17). PCR amplifications were performed as previously described (18). PCR products were separated on 3.5% polyacrylamide gels containing 7M urea, gels were dried and labelled products visualized by autoradiography. PCR products were subcloned and sequenced as previously described (16).

Construction of variant ER-α expression vector

The RT-PCR product corresponding to the exon 3/4 deleted ER-α cDNA was cloned into the TA cloning vector (Invitrogen TA cloning kit). Stu I digestion of this plasmid released an exon 3/4 deleted fragment which was used to replace the corresponding region of wild-type ER-α from p0R8 (19) (contains a glycine to valine point mutation at amino acid 400). Stu I sites are in exon 2 and 7 of wild-type ER-α and the subcloned d3/4 PCR fragment resulted in a correction to the wild-type sequence of glycine at amino acid 400 (in exon 5 in pOR8). The full length EcoR I ER-α fragment from HEGO (an expression plasmid containing wild-type ER-α coding region cloned into the eukaryotic expression vector pSG5, a gift from Dr. P. Chambon, Strasbourg, France (19)) was then excised and replaced with the corresponding fragment from pOR8 containing the exon 3/4 deleted ER-α cDNA. The identity of the expression plasmid containing the exon 3/4 deleted ER-α (called d3/4) was confirmed by restriction enzyme digest and sequence analysis.

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega, Madison, WI). Reactions were performed according to the manufacturer's instructions.

Western blotting and immune detection

Whole cell extracts (dissolved in 8M urea) were analysed using 10% SDS-PAGE with a 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method (20). Gels were transferred to nitrocellulose using CAPS transfer buffer (10 mM CAPS, pH 11, 20% methanol) and transferred for 1 hr at 120 V at 4°C. Blots were blocked for 1 hr at room temperature in 5% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were incubated with either: ER-α specific primary antibody, H226, (a generous gift from Dr G. Greene, University of Chicago, IL which recognizes an epitope in exon 1/exon 2 region of the wild-type ER-α) or the ER-α specific antibody, AER 308, (Neomarkers, CA, which recognizes an epitope in exon 4 of the wild-type ER-α), overnight at 4°C in 1% skimmed milk/Tris-buffered saline containing 0.5% Tween-20. Blots were then incubated with the appropriate horse radish peroxidase conjugated secondary antibody for 1 hr at room temperature in 1% skim milk/Tris-buffered saline containing 0.5% Tween-20. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, England).

Statistical Analysis,

Statistical analyses of ER-α transcriptional activity and PR levels were performed using paired Student's t-test. Statistical analysis on the effects of d3/4 on ERE-tk-CAT activity in T5 cells was performed using Wilcoxon's rank sum test. Statistical analysis on the effects of d3/4 on ERE-tk-CAT activity in MDA-MB-231 and MCF10A1 cells was performed on natural log transformed data using paired Student's t-test. Statistical analyses were performed with the help of M. Cheang, University of Manitoba, Biostatistical Consulting Unit.

Results

Apparent ligand-independent (basal) activity of ER-α is increased in T5-PRF cells.

Previously, we have shown that T5-PRF cells are no longer growth responsive to estrogen in culture, while still retaining expression of the ER-α (8). To investigate further the mechanism(s) responsible for estrogen-nonresponsiveness in these cells, ER-α transcriptional activity was examined by transient transfection assays using an estrogen-responsive reporter gene. The histogram in figure 1A represents the fold difference in chloramphenicol acetyl transferase (CAT) activity between T5 and T5-PR[illegible] As expected, estrogen treatment increases CAT activity in T5 and to a lesser extent [illegible]RF cells, while the antiestrogen ICI 164,384 inhibits the estrogen induced transcriptional activity of the ER-α in both cell lines. In the absence of added estrogen there is a low basal ER-α activity in parental T5 cells, however in the estrogen-nonresponsive T[illegible]ells, the basal ER-α activity was 3.6 ± 0.5 (mean ± sem, n=7) fold higher than th[illegible] cells ($p<0.05$). Consistent with the increased basal CAT activity in T5[illegible] cells being mediated by ER-α, treating cells under basal conditions with ICI alone, completely abolished the increased basal transcriptional activity (Figure 1B).

Progesterone receptor levels are elevated in T5-PRF cells.

PR expression is a marker of ER-α activity (21), therefore we examined PR levels to determine if the increase in basal ER-α activity in T5-PRF cells was reflected in an endogenous estrogen-responsive gene. Under basal (i.e. no added estrogen) conditions the T5-PRF cells have significantly higher PR levels (~3 fold) than the parent T5 cells passaged twice in PRF/DMEM (as defined in Methods) before receptor assays (464 ± 20 fmol/10^6 cells vs 148 ± 40 fmol/10^6 cells, mean ± sem, n=3, see figure 2). Previously, we had shown that T5-PRF cells retained expression of ER-α which was approximately 50% of the levels seen in the parent T5 cells (8). Since we observed increased basal activity from both an estrogen-responsive reporter gene and an endogenous estrogen-responsive gene (PR) in T5-PRF cells despite a decreased level of endogenous ligand-binding ER, we reasoned that the

intrinsic activity of the wild-type ER in these cells was increased or some ER-like activity existed that was not detectable by ligand binding experiments.

Expression of a variant ER-α mRNA deleted in exons 3 and 4 is increased in T5-PRF cells.

Alterations in the structure or presence of variant forms of the ER-α with ligand-independent activity could be one mechanism for our observed results. Long-range ER-α RT-PCR analysis (18) was performed on RNA isolated from T5 and T5-PRF cells to examine the pattern of deleted variant ER-α mRNA expression. RT-PCR analysis was performed, using a primer pair specific for exons 1 and 8 of wild-type human ER-α sequence, allowing detection of any variant ER-α mRNA species containing both exons 1 and 8 of wild-type ER-α sequence. Figure 3A shows the PCR products obtained and the presence of a 928 bp band that is markedly increased in T5-PRF estrogen-independent cells. To confirm the identity of this variant, the cDNA corresponding to the 928 bp band was subcloned and sequenced. The nucleotide sequence of the cDNA was found to represent a variant ER-α mRNA containing a deletion of both exons 3 and 4 (Figure 3B). The exon 3 and 4 deleted ER-α (d3/4) is in frame and is predicted to encode a protein of 443 amino acid residues with a predicted molecular mass of ~49 kDa. This putative ER-α-like protein would be missing the second zinc finger of the ER-α DNA binding domain, the hinge region and part of the ligand binding domain.

The exon 3/4 deleted ER-α-like protein increases basal and estrogen-regulated wild-type ER-α transcriptional activity.

To address the potential function of this variant ER-α mRNA eukaryotic expression vectors containing d3/4 cDNA were constructed and shown to express a protein of the appropriate size (Figure 4A), that was recognized by the ER-α antibody H226 that recognizes an epitope encoded in exon 1/2 (A/B region) of wild-type ER-α (Figure 4B, lanes 1 and 2). Using an antibody that recognizes an epitope encoded in exon 4 of the wild-type ER-α the band corresponding to the d3/4 protein is not seen, while wild-type ER-α is still detected (Figure 4B, lanes 3 and 4). Ligand binding analysis of the *in vitro* translated d3/4 protein showed little or no ability to bind estradiol specifically (data not shown). This

protein is missing the second zinc finger of the DNA binding domain and as such would not be expected to bind to DNA. We found that under conditions in which *in vitro* transcribed/translated wild-type ER-α could bind to an oligonucleotide containing the vitellogenin B1 ERE, d3/4 did not demonstrate any specific DNA binding in gel mobility shift assays (data not shown).

To examine the transcriptional activity of d3/4 variant ER-α, transient transfections using ER negative cell lines were carried out. Under conditions in which transiently transfected wild-type ER-α was transcriptionally active and able to induce CAT activity in a ligand-dependent fashion, the d3/4 ER-α did not demonstrate any transcriptional activity on its own (Figure 6A and 6B). This is unlikely to be due to low levels of expression of this transgene, since after transfection of 5 μg of d3/4 vector into MCF10A1 cells we were able to detect a protein corresponding in size to the expected d3/4 protein (Figure 5). To determine if d3/4 ER-α and wild-type ER-α could interact to influence transcription, cotransfections of wild-type and d3/4 ER-α into MDA-MB-231 and MCF10A1 ER negative breast epithelial cell lines were carried out (Figure 6A and B). HEGO transfected alone showed the expected estrogen-dependent activity while d3/4 alone had no transcriptional activity (Figure 6A and B). However, when increasing amounts of d3/4 ER-α were transfected with a constant amount of HEGO, d3/4 could increase both the basal and estrogen-dependent activity of wild-type ER-α. When equal amounts of d3/4 and wild-type HEGO were transfected into MDA-MB-231 and MCF10A1 cells a significant increase in the estrogen-dependent activity was seen. Increasing amounts of cotransfected d3/4 was associated with statistically significant increases in basal transcription in both MCF10A1 and MDA-MB-231 cells. In MDA-MB-231 cells when 1 pmol of d3/4 was transfected with 0.5 pmol of HEGO a statistically significant increase in basal transcription was observed, while in MCF10A1 cells we saw a statistically significant increase in basal transcription when 2 pmol of d3/4 was transfected with 0.5 pmol of HEGO.

We next examined the effects of introducing the d3/4 ER-α into the parental T5 cells. Transient transfection of d3/4 into T5 cells was carried out and ER-α transcriptional

activity measured. Figure 7 shows the results obtained, and demonstrates that transfection of 1 pmol of d3/4 caused a significant increase in CAT activity both in the presence and absence of added estrogen, despite the fact that this variant ER-α does not bind appreciably to ligand *in vitro* nor has transcriptional activity of its own at this concentration (Figure 6A and 6B).

Discussion

Numerous studies have identified variant ER-α mRNAs in both normal and neoplastic breast tissue and cell lines (16,18,22-25). While still a controversial topic, evidence is emerging to support the existence of ER-α variant proteins, which could correspond to some ER-α variant mRNAs, in some cell lines and tissues *in vivo* (22,26-32). However, the pathophysiological significance of ER-α variant expression is unclear. Altered expression of some ER-α variant mRNAs was found associated with both breast tumorigenesis and breast cancer progression (16,22,33-35). Several studies, using transient transfection analyses, have shown that individual ER-α variant proteins can have both positive and negative effects on wild-type ER-α activity (22,26,27,36-40). Conflicting results for some ER-α variants have been obtained (39,41) which may be due to cell and promoter specific events previously identified for various structure/function domains of the wild-type ER-α (42,43). Similarly, overexpression of a single ER-α variant using stable transfection technology has given different results in different laboratories (36,44). Moreover, direct correlation of any single ER-α variant with clinical tamoxifen resistance or tamoxifen resistance of breast cancer cells in culture has not been forthcoming. Since most of these comparisons have been performed using individual ER-α variants and do not take into account the entire spectrum of ER-α variants relative to each other, the conclusions remain controversial. Together, the data support the hypothesis that the development of hormone-independence and endocrine resistance in human breast cancer is a multifactorial process and indeed there are many examples where the development of estrogen-independent growth and antiestrogen resistance are dissociable events in breast cancer cell line models (45-48). Similar to these and other studies, we have found that the development of estrogen-independent growth in a breast cancer cell line model, through long term growth in estrogen-depleted medium, was not associated with antiestrogen resistance. Although the estrogen-nonresponsive T5-PRF cells have a reduced sensitivity to the pure antiestrogen ICI 164,384, their growth response to 4-hydroxytamoxifen is

identical to parental T5 cells (8). However, when we investigated the relative pattern of expression of ER-α deleted variant mRNA in T5-PRF compared to parental T5 cells, there was a significant difference in the relative expression of a previously described exon 3 plus 4 deleted ER-α variant mRNA (reviewed in 49). Although the question of whether this ER-α variant is a cause of estrogen independence or merely an effect of the selection process for estrogen independence requires further study, our data, using transient transfection analyses tend to support a possible functional role for the putative 3/4 deleted ER-α protein encoded by the variant mRNA in the phenotype observed in T5-PRF human breast cancer cells. In this study we have shown that T5-PRF cells have significantly increased ligand-independent (basal) ER-α activity (reflected in both ERE-tk-CAT activity and endogenous PR levels). The d3/4 variant ER-α was able to confer increased ligand-independent (basal) and estrogen-responsive transcriptional activity when expressed in parental T5 cells and when coexpressed with wild-type ER-α in ER-α negative human breast cell lines. While the demonstrated effect of d3/4 to increase HEGO basal transcriptional activity in the ER-α negative cell lines suggests a putative functional role for this variant ER-α, this effect required 2-4 times higher levels of d3/4 than HEGO. Although such data provide 'proof of principle' that the d3/4 ER-α variant can modulate both the ligand dependent and independent transcriptional activity of wild type ER-α, the relevance of the expression levels of each protein achieved in the reconstituted transient expression system to the endogenous levels of ER-α and d3/4 ER-α variant expression in T5-PRF is unclear. Furthermore, differences in the background of transcriptional co-activators and co-repressors between naturally ER-α positive and negative cell lines (for example, 43), as well as the presence of other naturally occurring ER-α variants in naturally ER-α positive cell lines are all likely to impact on the final outcome of ER mediated transcriptional activity and underlie the differences seen between the transiently manipulated cells and the naturally occurring T5-PRF phenotype. Moreover, expression of ER-β and/or its variants may influence estrogen action (50). Both T5 and T5-PRF cells express low levels of ER-β mRNA determined by reverse transcription polymerase chain

reaction analysis (51, unpublished data), however, the functional significance of the levels remains unknown. Nonetheless, we saw a significant effect on ER-α ligand-independent transcription in T5 cells at levels of co-transfected d3/4 that likely would not be higher than the endogenous ER-α in these cells, but the extrapolation of these data to the relative expression of wild-type ER-α and d3/4 ER-α variant in T5-PRF cells is presently unknown. It is of significance that we can reproduce an effect of this d3/4 variant in the parental T5 cells, which would likely contain a more representative background of ER-α accessory proteins (ie. co-activators and/or co-repressors) as well as other variant forms of ER-α which would all contribute to the final ER mediated biological response. As well, our data do not exclude the possibility that other alterations have occurred in T5-PRF cells which, in combination with an altered ER-α variant, may contribute to the estrogen-independent phenotype of T5-PRF cells.

It has previously been shown that breast cancer cells can adapt to low levels of estrogen by enhancing their sensitivity to estrogen (52). Estrogen-deprivation of MCF-7 human breast cancer cells resulted in estrogen hypersensitivity and maximal growth was achieved with an estrogen concentration 4-5 orders of magnitude lower than wild-type cells. These researchers also found that the concentration of ICI needed to inhibit growth of these cells was ~6 orders of magnitude lower than wild-type cells, supporting the hypothesis in this model, that increased sensitivity to ER ligands had occurred. While supersensitivity to estrogen in T5-PRF cells cannot be entirely ruled out, we have previously shown that, while T5-PRF cells are sensitive to growth inhibition by ICI 164,384, in contrast to the data of Masamura *et al* , they are less sensitive than the parental T5 cells (i.e. ID_{50} 100 nM and 5μM for T5 and T5-PRF, respectively), suggesting that in this model other mechanisms are likely involved.

Our data do not address the mechanism by which d3/4 enhances ER transcriptional activity, but several possibilities exist. The ER-α contains at least two separate regions that are required for optimal transcriptional activation (42,43). The amino-terminal region contains promoter and cell-type specific ligand-independent transcriptional activity (AF1)

and the second, AF2, is located in the ligand-binding carboxyl-terminus of the receptor. Exon 3/4 deleted ER-α containing an intact AF2 or AF1 domain could interfere with, or sequester an ER-α repressor protein resulting in increased ER-α transcriptional activity in the absence of ligand (53). This variant may also retain the ability to interact with other ER-α regulatory proteins such as coactivators or components of the basal transcription machinery.

ER-α also contains two domains involved in dimerization (54,55). A weak dimerization interface is present in the DNA-binding domain (DBD) and a strong interface is located in the C-terminal ligand-binding domain (56). d3/4 containing an intact C-terminal dimerization domain, may form heterodimers with wild-type ER-α that have altered transcriptional regulatory properties through differing protein-protein interactions.

The crystal structure of the ER-α hormone binding domain has recently been elucidated (57). Based on this structure, the d3/4 protein would contain many of the regions essential for transactivation, including the predominant helix 12 (encompassing amino acids 539-547). However, since d3/4 alone has no transcriptional activity (at least on a classical ERE regulated promoter) the structure must be sufficiently altered to prevent activity, or AF2 can only be activated in a ligand-dependent manner but d3/4 cannot bind ligand. Helix 12 in AF2 is believed to be the main region involved in coactivator recruitment and it may be possible that d3/4, following heterodimerization with ER-α, enhances recruitment of coactivators to the basal transcription complex and this enhances ER-α activity.

We have found that using a transient expression system, the d3/4 ER-α caused increased ligand-independent wild-type ER-α activity and also enhanced the ligand-induced ER-α transcriptional activity, despite the fact that on its own this variant is not transcriptionally active on a classical ERE promoter, nor does it bind ligand *in vitro* to any significant degree. Studies have demonstrated that the ability of steroid hormone receptors to modulate transcription does not necessarily require that the receptors bind DNA. PRc, an N-terminally truncated PR isoform lacking the first zinc-finger of the DBD, has no

transcriptional activity of its own but has been shown to enhance progestin-induced transcriptional activity (58). The DBD of the ER-α does not appear to be necessary for raloxifene activation of the TGFβ3 gene (59) and ER-α can activate transcription from AP-1 dependent promoters through a DNA binding-independent pathway (60). Sp1 and ER-α directly interact to enhance estrogen-induced transactivation of the Sp1-dependent Hsp27 gene promoter and the DBD of the ER-α is not required (61).

Recent research has demonstrated that the ER-α can be activated in a ligand-independent fashion (62-64). Studies have shown that several growth factors such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and insulin-like growth factor (IGF-1) were able to activate the ER-α in the absence of ligand. The ability to activate the ER-α in the absence of estrogen could confer a growth advantage to breast cancer cells and aid in the development of a hormone-independent phenotype. The presence of alternate forms of ER-α capable of interacting with wild-type ER-α to increase ligand-independent activity could also confer a potential growth advantage to breast cancer cells. A recent study has shown that constitutively active, ligand-independent ER-α mutants undergo conformational changes and interactions with coactivators that mimic changes in ER-α that are usually regulated by ligand (65). Recently, researchers have shown that TR-β2 is a ligand-independent activator of the gene encoding thyrotropin-releasing hormone (TRH) and have mapped a region in the N-terminus of the receptor responsible for this activity (66). These researchers suggest that the mechanism of ligand-independent activation involves direct interaction of the TR-β2 amino terminus with either transcriptional cofactors or the basal transcription machinery itself.

An increased relative expression of variant ER-α proteins containing intact AF domains, could result in increased interactions with the ER-α and/or other proteins involved in ER-α transcriptional activity. This could be a potential mechanism for estrogen-independent growth associated with the presence of one or more variant ER species and could explain the increased ER-α activity we have seen with the d3/4 ER-α.

Acknowledgements

This work was supported by the U.S. Army Medical Research and Material Command (USAMRMC). LCM is a Medical Research Council of Canada (MRC) Scientist, EL is a recipient of a USAMRMC Postdoctoral Fellowship, and AC holds a Manitoba Health Research Council (MHRC) Studentship. The authors also wish to acknowledge the generous contribution of the ER antibody, H226, from Dr. G. Green, University of Chicago, IL.

FIGURE LEGENDS

Figure 1. ER transcriptional activity. A. T5 and T5-PRF cells were transfected and CAT assays performed as described in Methods. Results are expressed as fold CAT activity compared to T5 basal (arbitrarily set at 1.0). *$p<0.05$, Student's t-test (compared to T5 basal). Results represent mean $\pm$ SEM, n=7. **B.** T5 and T5-PRF cells were transfected and CAT assays performed as described in Methods. T5-PRF cells were treated with ICI 164,384 alone under basal (estrogen-free) conditions. Results represent fold CAT activity as compared to T5 basal, n=2.

Figure 2. Basal progesterone receptor levels. PR levels were determined by whole cell binding as described in Methods. PR levels are expressed as fmol PR/10^6 cells and results represent mean $\pm$ SEM, n=3, **$p<0.01$, Student's t-test.

Figure 3. Identification of exon 3/4 deleted variant ER-α mRNA. A. Long-range ER-α RT-PCR. Total RNA was extracted from T5 and T5-PRF cells, reverse transcribed and PCR amplified using 1/8U and 1/8L primers. Labelled PCR products were separated on 3.5% acrylamide-urea gels and visualized using autoradiography. WT-ER = wild type ER-α, D7-ER = exon 7 deleted ER-α, D4-ER = exon 4 deleted ER-α and D3/4-ER = exon 3 and 4 deleted ER-α, based on size compared to labelled markers (not shown). **B.** Sequence of d3/4 ER-α cDNA. The 928bp PCR product was excised from a gel and subcloned (in triplicate) and three colonies from each independent subcloning were sequenced to confirm the identity of d3/4 cDNA.

Figure 4. *In vitro* transcription/translation and Western blotting of d3/4 protein. A. Expression vectors containing wild-type ER-α (lane 1) and d3/4 cloned into pSG5 (lane 2) were subjected to *in vitro* transcription/translation reactions in the presence of ^{35}S-methionine. Reaction products were run on 10% SDS-acrylamide gel, dried and exposed to film overnight. **B.** *In vitro* transcription/translation products were run on 10% SDS-acrylamide gels, transferred to nitrocellulose and Western blotting performed. Lanes 1 and 2 were visualized

using ER-α Ab H226 (epitope located in ER-α exon 1/2) and lanes 3 and 4 using ER-α Ab 308 (epitope located in ER-α exon4).

Figure 5. *In vivo* expression of d3/4 protein. MCF10A1 human breast epithelial cells were transfected with the appropriate expression vector, cells were lysed in 8M urea, and 10 μg protein run on a 10% SDS-acrylamide gel. Gels were transferred to nitrocellulose and Western blotting performed using ER-α Ab H226. Lane 1. Control cells transfected with 5 μg of vector (pSG5) alone; Lane 2. Cells transfected with 5 μg HEGO (WT-ER-α) expression vector; Lane 3. Cells transfected with 5 μg d3/4 expression vector; Lane 4. *In vitro* transcribed/translated WT-ER-α (1 μl); Lane 5. *In vitro* transcribed/translated d3/4 ER-α (2 μl).

Figure 6. Activity of d3/4 in ER-α negative cells. A. MDA-MB-231 cells were transfected with 5μg ERE-tk-CAT, 1μg pCH110, 0.5pmol HEGO ± 0.5-2pmol d3/4 ± vector DNA to give a total of 17 μg DNA/dish . Cells were treated with 10nM estradiol (E2) for 24h or vehicle alone as control. Results are expressed as fold CAT activity compared to basal HEGO activity arbitrarily set as 1. Histograms represent mean ± SEM, n=5-7. *b=p<0.05 paired Student's t-test, result compared to basal HEGO alone; *e=p<0.05 paired Student's t-test, result compared to estradiol treated HEGO alone. **B**. MCF10A1 cells were similarily transfected. Histograms represent mean ± SEM, n=4. *b=p<0.05 paired Student's t-test, result compared to basal HEGO alone, *e=p<0.05, paired Student's t-test, result compared to estradiol treated HEGO alone.

Figure 7. Transient transfection of d3/4 expression vector into T5 cells. Cells were grown in PRF-DMEM as described in Methods and transfected with 5μg ERE-tk-CAT expression vector, 5 μg pCH110 along with the appropriate amount of d3/4 expression vector. Cells were treated with vehicle or 10nM estradiol (E2) for 24h, harvested and CAT assays performed. Results represent mean ± SEM, n=2-5, *b=p<0.05 Wilcoxon's rank sum test, result compared to basal ERE-tk-CAT activity, *e=p<0.05 Wilcoxon's rank sum compared to estradiol treated ERE-tk-CAT activity.

References

1. Dickson RB. 1991 Growth regulation of normal and malignant breast epithelium. In: The breast: comprehensive management of benign and malignant diseases. pp 363-394.
2. Nandi S, Guzman RC, Yang J. 1995 Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci U S A. 92:3650-3657.
3. Merkel DE, Osborne CK. 1989 Prognostic factors in breast cancer. Hematol Oncol Clin North Am. 3:641-652.
4. Leonessa F, Boulay V, Wright A, Thompson EW, Brunner N, Clarke R. 1992 The biology of breast tumor progression. Acquisition of hormone independence and resistance to cytotoxic drugs. Acta Oncol. 31:115-123.
5. Clarke R, Dickson RB, Brunner N. 1990 The process of malignant progression in human breast cancer. Ann Oncol. 1:401-407.
6. Horwitz KB. 1993 Mechanisms of hormone resistance in breast cancer. Breast Cancer Res Treat. 26:119-130.
7. Earp HS, Dawson TL, Li X, Yu H. 1995 Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. Breast Cancer Res Treat. 35:115-132.
8. Coutts AS, Davie JR, Dotzlaw H, Murphy LC. 1996 Estrogen regulation of nuclear matrix-intermediate filament proteins in human breast cancer cells. J Cell Biochem. 63:174-184.
9. Watts CKW, Handel ML, King RJB, Sutherland RL. 1992 Oestrogen receptor gene structure and function in breast cancer. J Steroid Biochem Molec Biol. 41:529-536.
10. Soule HD, Maloney TM, Wolman SR, et al. 1990 Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50:6075-6086.
11. Murphy LC, Dotzlaw H. 1989 Endogenous growth factor expression in T-47D, human breast cancer cells, associated with reduced sensitivity to antiproliferative effects of progestins and antiestrogens. Cancer Res. 49:599-604.

12. Graham FL, van der Eb A. 1973 A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology. 52:456-467.

13. Kingston R. 1989 Harvest and assay for chloramphenicol acetyltransferase. Current protocols in molecular biology 1:963-966.

14. Rosenthal N. 1987 Identification of regulation elements of cloned genes with functional assay. Methods Enzymology: Guide to molecular cloning techniques. 152:704-720.

15. Seiler-Tuyns A, Walker P, Martinez E, Merillat AM, Givel F, Wahli W. 1986 Identification of estrogen-responsive DNA sequences by transient expression experiments in a human breast cancer cell line. Nucleic Acids Res. 14:8755-8770.

16. Leygue ER, Watson PH, Murphy LC. 1996 Estrogen receptor variants in normal human mammary tissue. J Natl Cancer Inst. 88:284-290.

17. Green S, Walter P, Kumar V, et al. 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v- erb-A. Nature. 320:134-139.

18. Leygue E, Huang A, Murphy LC, Watson PH. 1996 Prevalence of estrogen receptor variant messenger RNAs in human breast cancer. Cancer Res. 56:4324-4327.

19. Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I, Chambon P. 1989 The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. Embo J. 8:1981-1986.

20. Laemmli UK. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

21. Horwitz KB, McGuire WL. 1978 Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. J Biol Chem. 253:2223-2228.

22. Fuqua SA, Fitzgerald SD, Allred DC, et al. 1992 Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. Cancer Res. 52:483-486.

23. Garcia T, Lehrer S, Bloomer WD, Schachter B. 1988 A variant estrogen receptor messenger ribonucleic acid is associated with reduced levels of estrogen binding in human mammary tumors. Mol Endocrinol. 2:785-791.

24. Murphy LC, Dotzlaw H. 1989 Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. Mol Endocrinol. 3:687-693.

25. McGuire WL CG, Fuqua SA. 1992 Abnormal estrogen receptor in clinical breast cancer. J Steroid Biochem Mol Biol. 43:243-347.

26. Castles CG, Fuqua SA, Klotz DM, Hill SM. 1993 Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. Cancer Res. 53:5934-5939.

27. Pink JJ, Jiang SY, Fritsch M, Jordan VC. 1995 An estrogen-independent MCF-7 breast cancer cell line which contains a novel 80-kilodalton estrogen receptor-related protein. Cancer Res. 55:2583-2590.

28. Moncharmont B, Ramp G, De Goeij CC, Sluyser M. 1991 Comparison of estrogen receptors in hormone-dependent and hormone- independent Grunder strain mouse mammary tumors. Cancer Res. 51:3843-3848.

29. Montgomery PA, Scott GK, Luce MC, Kaufmann M, Benz CC. 1993 Human breast tumors containing non-DNA-binding immunoreactive (67 kDa) estrogen receptor. Breast Cancer Res Treat. 26:181-189.

30. Raam S, Robert N, Pappas CA, Tamura H. 1988 Defective estrogen receptors in human mammary cancers: their significance in defining hormone dependence. J Natl Cancer Inst. 80:756-761.

31. Marsigliante S, Baker VA, Puddefoot J, Barker S, Vinson GP. 1991 4 S oestrogen receptor isoforms and their distribution in breast cancer samples. J Mol Endocrinol. 7:205-211.

32. Scott GK, Kushner P, Vigne JL, Benz CC. 1991 Truncated forms of DNA-binding estrogen receptors in human breast cancer. J Clin Invest. 88:700-706.

33. Leygue E, Murphy L, Kuttenn F, Watson P. 1996 Triple primer polymerase chain reaction. A new way to quantify truncated mRNA expression. Am J Pathol. 148:1097-103.

34. Daffada AA, Johnston SR, Smith IE, Detre S, King N, Dowsett M. 1995 Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance

and progesterone receptor/pS2 status in human breast cancer. Mol Cell Endocrinol. 109:197-207.

35. Murphy LC, Hilsenbeck SG, Dotzlaw H, Fuqua SAW. 1995 Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. Clin Cancer Res. 1:155-159.
36. Rea D, Parker MG. 1996 Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. Cancer Res. 56:1556-1563.
37. Desai AJ, Luqmani YA, Walters JE, et al. 1997 Presence of exon 5-deleted oestrogen receptor in human breast cancer: functional analysis and clinical significance. Br J Cancer. 75:1173-1184.
38. Fuqua SA, Fitzgerald SD, Chamness GC, et al. 1991 Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res. 51:105-109.
39. Wang Y, Miksicek RJ. 1991 Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol. 5:1707-1715.
40. Pink JJ, Wu SQ, Wolf DM, Bilimoria MM, Jordan VC. 1996 A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. Nucleic Acids Res. 24:962-969.
41. Fuqua SA, Allred DC, Elledge RM, et al. 1993 The ER-positive/PgR-negative breast cancer phenotype is not associated with mutations within the DNA binding domain. Breast Cancer Res Treat. 26:191-202.
42. Tora L, White J, Brou C, et al. 1989 The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell. 59:477-487.
43. Tzukerman MT, Esty A, Santiso-Mere D, et al. 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol. 8:21-30.
44. Fuqua SA, Wolf DM. 1995 Molecular aspects of estrogen receptor variants in breast cancer. Breast Cancer Res Treat. 35:233-241.
45. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. 1993 Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of

estrogen-regulated genes but without detectable DNA amplifications. Cancer Res. 53:283-290.

46. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. 1987 Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long- term absence of estrogens. Cancer Res. 47:4355-4360.
47. Welshons WV, Jordan VC. 1987 Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. Eur J Cancer Clin Oncol. 23:1935-1939.
48. van den Berg HW, Martin J, Lynch M. 1990 High progesterone receptor concentration in a variant of the ZR-75-1 human breast cancer cell line adapted to growth in oestrogen free conditions. Br J Cancer. 61:504-507.
49. Murphy LC, Dotzlaw H, Leygue E, Douglas D, Coutts A, Watson PH. 1997 Estrogen receptor variants and mutations. J Steroid Biochem Molec Biol. 62:363-372.
50. Giguere V. Tremblay A, Tremblay GB. 1998 Estrogen receptor-β : re-evaluation of estrogen and antiestrogen signaling. Steroids 63: 335-339.
51. Dotzlaw H, Leygue E, Watson PH, Murphy LC. 1997 Expression of estrogen receptor-*beta* in human breast tumors. J Clin Endocrinol Metabol. 82: 2371-2374.
52. Masamura S, Santner SJ, Heitjan DF, Santen RJ. 1995 Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab. 80:2918-2925.
53. Lee HS, Aumais J, White JH. 1996 Hormone-dependent transactivation by estrogen receptor chimeras that do not interact with hsp90. Evidence for transcriptional repressors. J Biol Chem. 271:25727-25730.
54. Kumar V, Chambon P. 1988 The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell. 55:145-156.
55. Schwabe JW, Chapman L, Finch JT, Rhodes D. 1993 The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell. 75:567-78.
56. White R, Fawell SE, Parker MG. 1991 Analysis of oestrogen receptor dimerisation using chimeric proteins. J Steroid Biochem Mol Biol. 40:333-341.

57. Brzozowski AM, Pike AC, Dauter Z, et al. 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. Nature. 389:753-758.

58. Wei LL, Hawkins P, Baker C, Norris B, Sheridan PL, Quinn PG. 1996 An amino-terminal truncated progesterone receptor isoform, PRc, enhances progestin-induced transcriptional activity. Mol Endocrinol. 10:1379-1387.

59. Yang NN, Venugopalan M, Hardikar S, Glasebrook A. 1996 Identification of an estrogen response element activated by metabolites of 17beta-estradiol and raloxifene [see comments] [published erratum appears in Science 1997 Feb 28;275(5304):1249]. Science 273:1222-1225.

60. Webb P, Lopez GN, Uht RM, Kushner PJ. 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol. 9:443-456.

61. Porter W, Saville B, Hoivik D, Safe S. 1997 Functional synergy between the transcription factor Sp1 and the estrogen receptor. Mol Endocrinol. 11:1569-1580.

62. Aronica SM, Katzenellenbogen BS. 1993 Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. Mol Endocrinol. 7:743-752.

63. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA. 1993 Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. Mol Endocrinol. 7:992-998.

64. Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA, Korach KS. 1996 Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. Endocrinology. 137:1735-1744.

65. Lazennec G, Ediger TR, Petz LN, Nardulli AM, Katzenellenbogen BS. 1997 Mechanistic aspects of estrogen receptor activation probed with constitutively active estrogen receptors: correlations with DNA and coregulator interactions and receptor conformational changes. Mol Endocrinol. 11:1375-1386.

66. Langlois MF, Zanger K, Monden T, Safer JD, Hollenberg AN, Wondisford FE. 1997 A unique role of the beta-2 thyroid hormone receptor isoform in negative regulation by thyroid hormone. Mapping of a novel amino- terminal domain important for ligand-independent activation. J Biol Chem. 272:24927-24933.

5

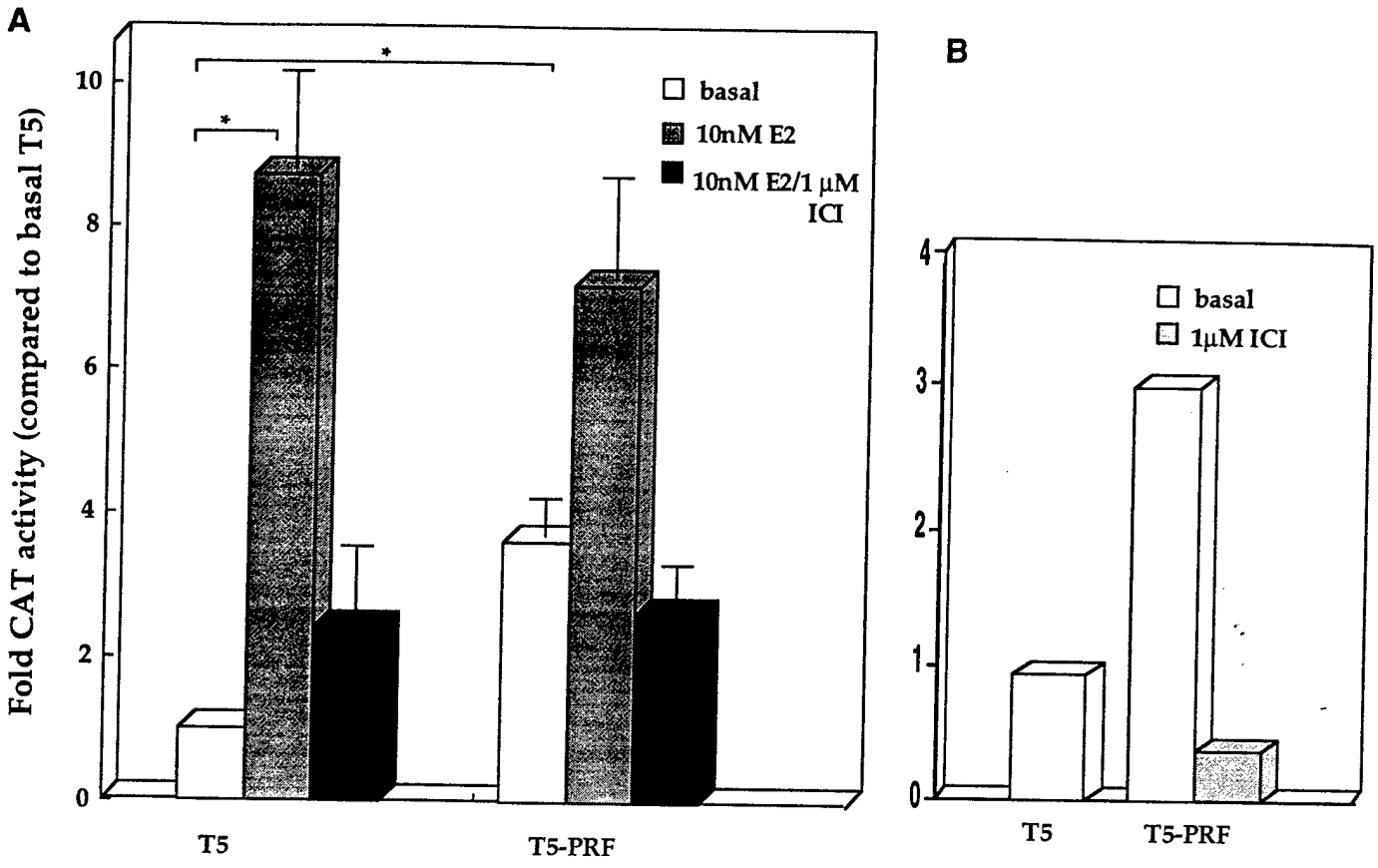

A
Fold CAT activity (compared to basal T5)
10
8
6
4
2
0
*
*
basal
10nM E2
10nM E2/1 μM ICI
T5
T5-PRF
B
4
3
2
1
0
basal
1μM ICI
T5
T5-PRF

Figure 1

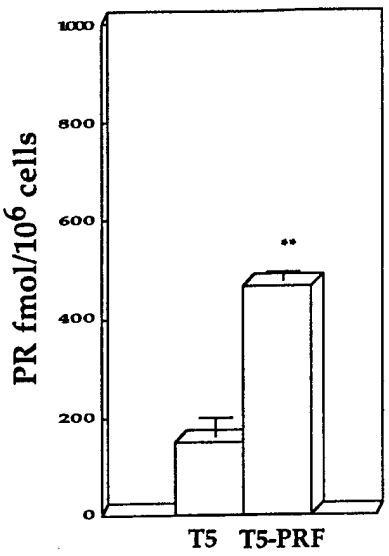


Figure 2.

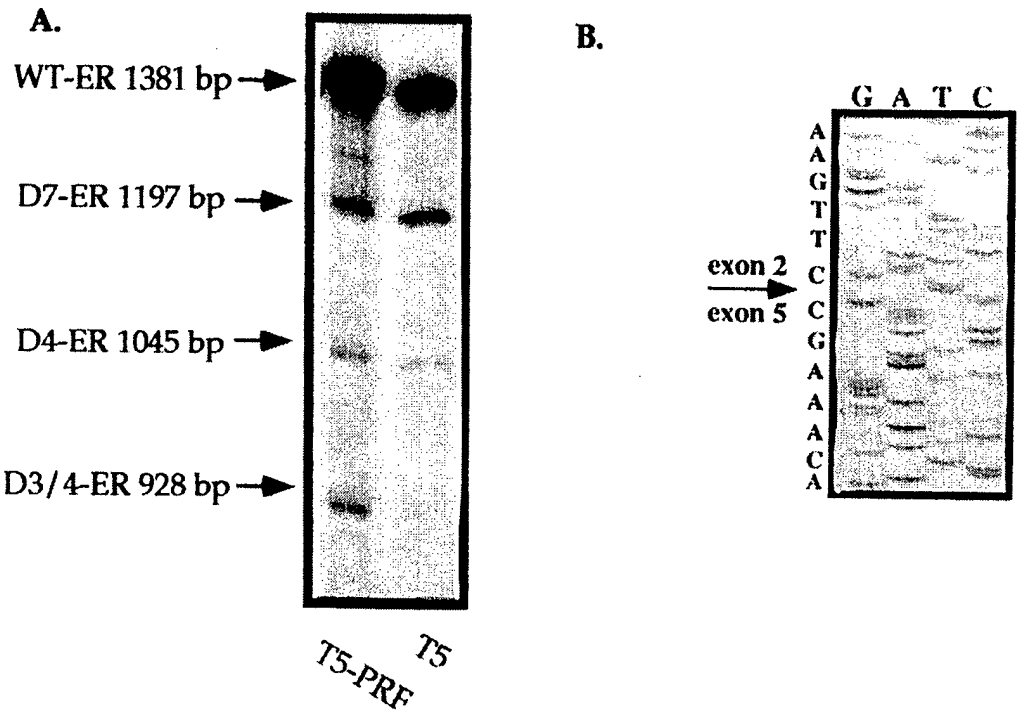

A.
WT-ER 1381 bp
D7-ER 1197 bp
D4-ER 1045 bp
D3/4-ER 928 bp
T5-PRF
T5
B.
G A T C
A
A
G
T
T
C
C
G
A
A
A
C
A
exon 2
exon 5

Fig 3

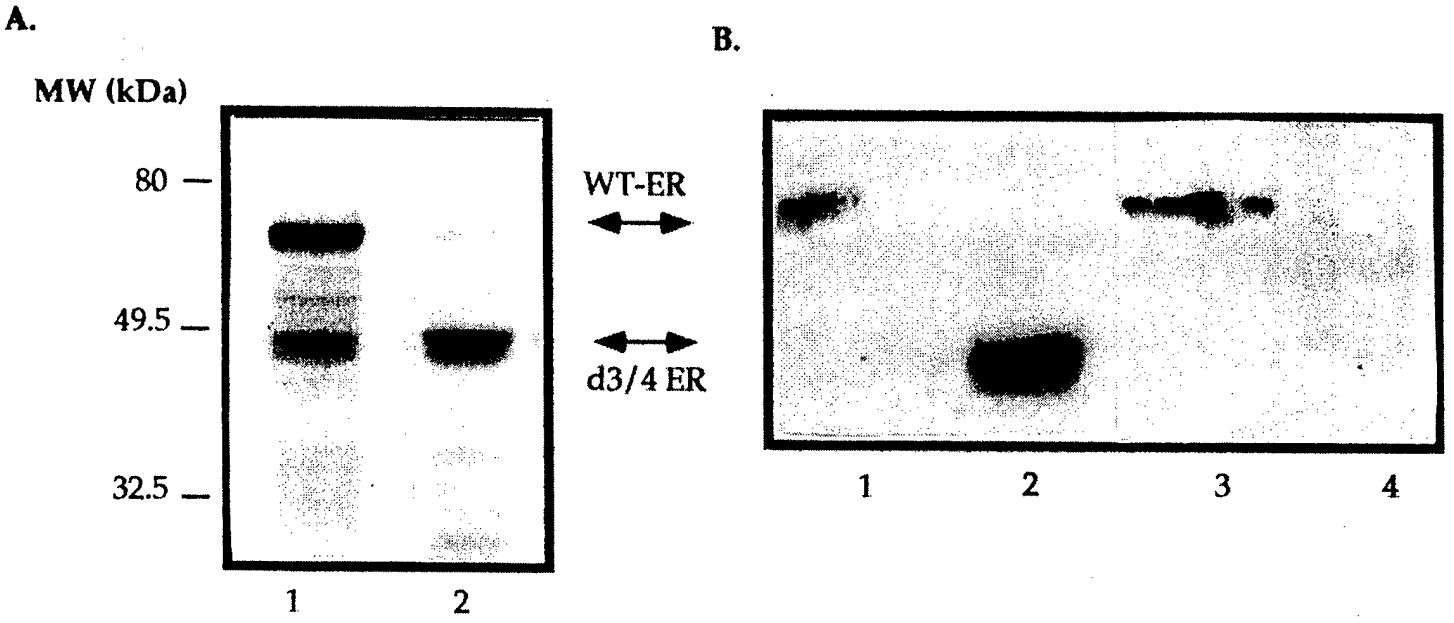


Fig 4

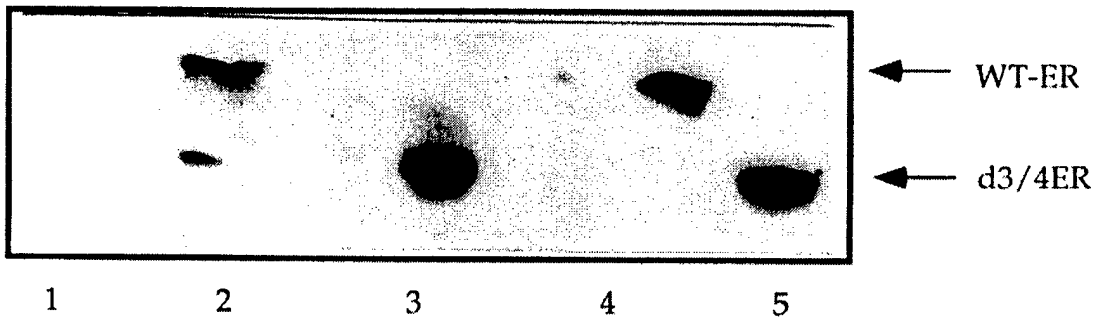


Fig 5

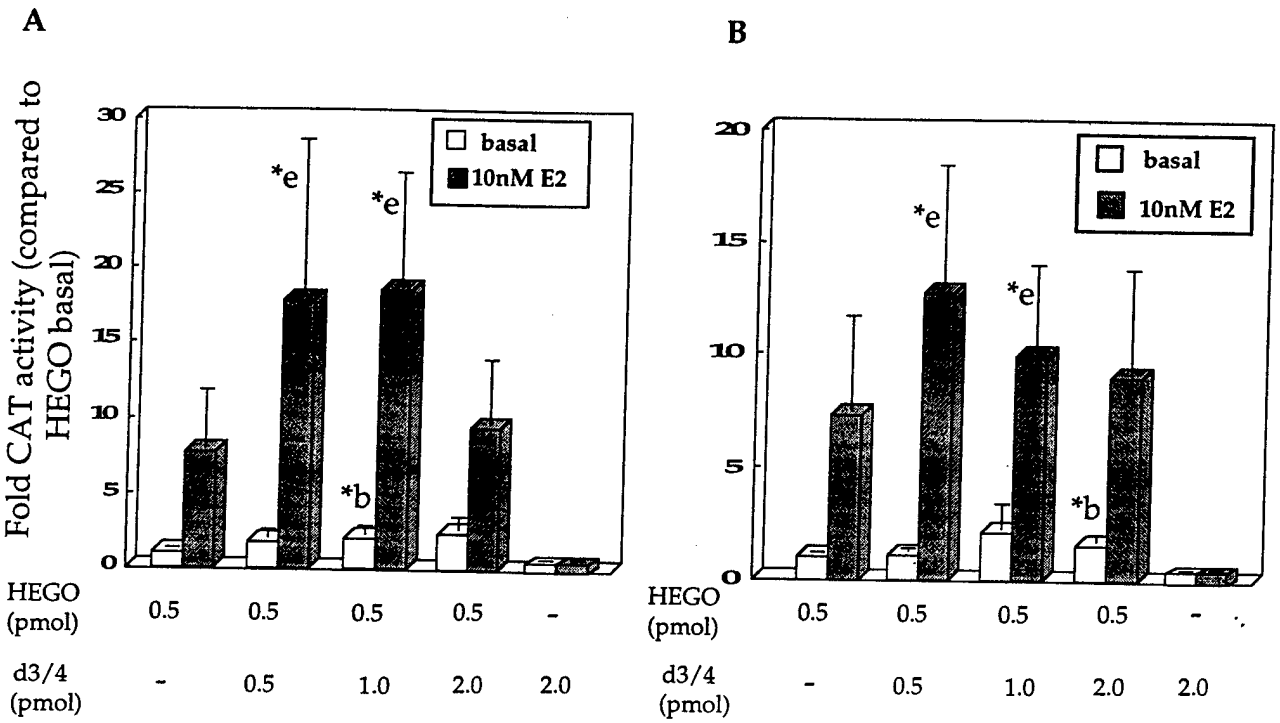

A
B
Fold CAT activity (compared to HEGO basal)
basal
10nM E2
*e
*b
HEGO (pmol)
d3/4 (pmol)
0.5
1.0
2.0
-

Figure 6.

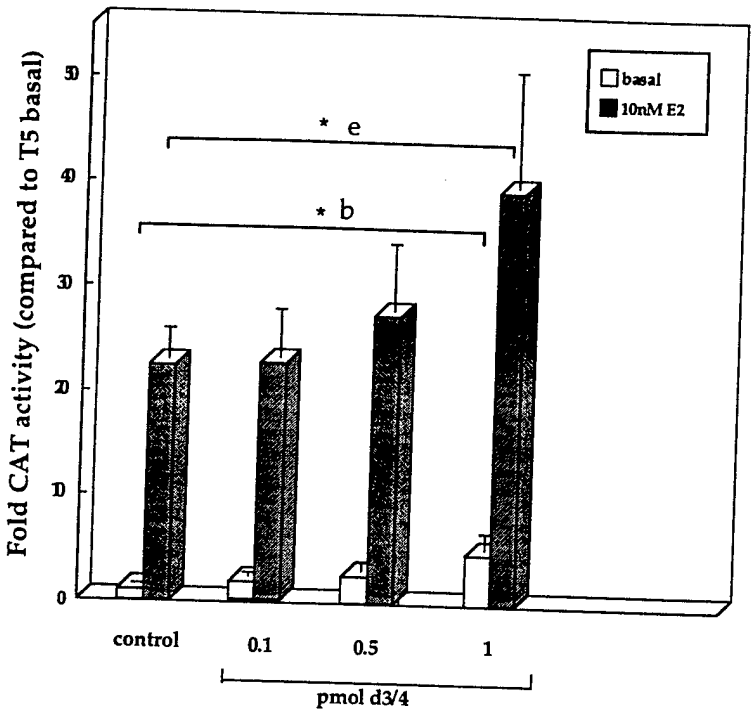

basal
10nM E2
* e
* b
Fold CAT activity (compared to T5 basal)
0
10
20
30
40
50
control
0.1
0.5
1
pmol d3/4

Figure 7.

APPENDIX 6

Chapter in Endocrine Oncology
S. Ethier (Ed) Humana Press

Multiple Facets of the Estrogen Receptor in Human Breast Cancer.

Leigh C Murphy, Etienne Leygue, Helmut Dotzlaw, Amanda Coutts, Biao Lu, Aihua Huang*, Peter H Watson*.

Dept of Biochemistry & Molecular Biology, and Dept of Pathology*, Faculty of Medicine, University of Manitoba, Winnipeg. Manitoba. Canada. R3E 0W3.

Telephone: 204-789-3233
Fax: 204-789-3900
E.mail: lcmurph@cc.umanitoba.ca

Abstract. The potential for multiforms of estrogen receptor like proteins to be expressed in human breast tissue is supported by the detection of estrogen receptor-α mRNA, estrogen receptor-β mRNA and their variant mRNAs in both normal and neoplastic human breast tissues. This suggests that studies of the interaction of estrogen and antiestrogen with human breast tissue must take into account the multifaceted nature of the estrogen receptor. Alterations in the expression of the mRNA of the various estrogen receptor facets during breast tumorigenesis and breast cancer progression suggest possible roles in these complex processes. However, there are still major gaps that need to be addressed before we have a clear idea of the pathophysiological and functional relevance of the experimental results so far published.

Introduction.

Estrogen is a major regulator of mammary gland development and function as well as affecting the growth and progression of mammary cancers (1,2). In particular the growth responsiveness of breast cancer cells to estrogen is the basic rationale for the efficacy of the so-called endocrine therapies such as antiestrogens. Estrogens mediate their action via the estrogen receptor (ER) which belongs to the steroid/thyroid/retinoid receptor gene superfamily (3). The protein products of this family are intracellular ligand-activated transcription factors regulating the expression of several gene products, which ultimately elicit a target tissue specific response (4). Indeed ER together with progesterone receptor (PR) expression in human breast tumors are important prognostic indicators as well as markers of responsiveness to endocrine therapies (5,6). However, although the majority of human breast cancers are thought to be initially hormone responsive it is well appreciated that alterations in responsiveness to estrogen must occur during breast tumorigenesis and in particular during breast cancer progression since several ER+ breast cancers are *de novo* resistant to endocrine therapies and of those that originally respond many develop resistance. This progression from hormonal dependence to independence, is a significant clinical problem since it limits the useful of the relatively non-toxic endocrine therapies and is associated with a more aggressive disease phenotype (7). This occurs despite the continued expression of ER and often PR (8,9). The ER is pivotal in estrogen and antiestrogen action in any target cell, however, the nature of the ER is now clearly multifaceted.

Until recently it was thought that only one ER gene existed. However a novel ER, now referred to as ER-β, has recently been cloned and characterized (10,11). Moreover, it has recently been shown that ER-β mRNA is expressed in both normal and neoplastic human breast tissue (12-14). This suggests that ER-β may have a role in estrogen action in both normal and neoplastic human breast tissue. Furthermore, it has now become apparent that several variant mRNA species of both the classical ER-α and ER-β can be expressed in human breast tissues and may therefore have roles in estrogen and antiestrogen signal transduction (13,15-18). The current data suggest that an evaluation of estrogen interaction with human breast tissue needs to include ER-α, ER-β and any variant forms of these receptors that may be

expressed. The following article focuses on this multifaceted nature of the ER in human breast tissues.

I. Estrogen Receptor-α and its Variants.

i. Identification of ER-α variant mRNAs in Human Breast Tissues. A large body of data has accumulated supporting the existence of ER-α variants (19,20). The majority of the data supporting the expression of ER-α variants has been at the mRNA level. Two main structural patterns of ER-α variant mRNAs have been consistently identified: the truncated ER-α mRNAs (21) and the exon deleted ER-α mRNAs (22). The truncated ER-α mRNAs were originally identified by Northern blot analysis as fairly abundant smaller sized mRNA species in some human breast cancer biopsy samples (23). The cDNAs of several truncated ER-α mRNAs have been cloned and found to contain authentic polyadenylation signals followed by poly A tails. The exon deleted ER-α mRNAs have been identified mainly from reverse transcription polymerase chain reaction (RT-PCR) products using targeted primers.

Multiple ER-α variant mRNAs are often detected in any one tumor sample. In order to determine the relative frequency and pattern of variant expression in any one sample an RT-PCR approach was developed which allowed the simultaneous detection of all deleted ER-α variant mRNAs containing the primer annealing sites in exons 1 and 8, at levels that represent their initial relative representation in the RNA extract. Since truncated transcripts do not have exon 8 sequences they will not be measured by this technique. Examples of the results obtained are shown (Figure 1) and serve to illustrate: I) that a complex pattern of exon deleted variant ER-α transcripts are expressed in any one tumor; ii) that the pattern and relative frequency of detection of ER-α variant mRNAs can vary between tumors, and iii) that in some cases the relative frequency of detection of individual ER-α variant mRNAs can be correlated with known prognostic markers (24).

An example of such a correlation is shown in Figure 2 (25). The expression of the truncated clone 4 ER-α variant mRNA was measured relative to the wild type ER-α mRNA in a group of breast tumors. The relative expression of the clone 4 variant was significantly increased in those tumors with characteristics of poor prognosis compared to those tumors with

good prognostic characteristics i.e. clone 4 expression was higher in large tumors with high S phase fraction and from patients with nodal involvement; compared to small tumors with low S phase fraction and from patients without nodal involvement. As well in this group the relative expression of clone 4 was significantly higher in PR negative tumors versus PR+ tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance.

The data supported the possibility that ER-α variant proteins might exist, their pattern and frequency changed amongst tumors, and in some cases the expression of single ER-α variant mRNA species was correlated with known markers of prognosis and endocrine sensitivity. Which in turn suggested the hypothesis that altered expression of ER-α variants could be a mechanism associated with hormonal progression in breast cancer.

ii. Putative Biological Significance of ER-α variant mRNAs. Important issues associated with the biological significance of ER-α variant mRNAs are whether they are tumor specific and the presence of proteins corresponding to those predicted from the variant mRNAs.

iia. The Expression of ER-α variant mRNAs in Normal and Neoplastic Human Breast Tissue. Most studies investigating ER-α variant mRNAs have used human breast cancer tissues or cell lines (19). However, it is now known that both truncated and exon deleted ER-α variant mRNAs can be detected in other tissues including normal, non-neoplastic tissues (19). In particular ER-α variant mRNAs have been identified in normal human breast tissue and cells (26-29). Therefore, ER-α variant mRNAs are not tumor specific, are not found in the complete absence of the wild type ER-α mRNA and are most likely generated by alternative splicing mechanisms.

This raised the question of whether the expression of ER-α variant mRNAs is altered during breast tumorigenesis and/or progression. When the level of expression of individual variant ER-α mRNAs was measured relative to the level of the wild type ER transcript differences between normal and breast tumor tissues were found. The relative expression of clone 4 truncated ER-α variant mRNA and the exon 5 deleted ER-α variant mRNA but not the

exon 7 deleted ER-α variant mRNA was significantly increased in breast tumors compared to normal breast tissues obtained from both reduction mammoplasties and normal tissues adjacent to breast tumors (26,27). Preliminary data suggests that this is also true for samples of ER positive breast tumors and their matched, adjacent normal tissues (Leygue *et al.,* unpublished data). As well, there is evidence suggesting that an exon 3 deleted ER-α variant mRNA is decreased in breast cancers compared to normal human breast epithelium (29). Since this ER-α variant mRNA encodes a protein which can inhibit wild type ER-α transcriptional activity (30) and cause growth suppression when stably overexpressed in ER+ MCF-7 human breast cancer cells (29), it was concluded that the exon 3 deleted ER-α variant may function to attenuate estrogenic effects in normal mammary epithelium, a function markedly reduced via decreased exon 3 deleted ER-α expression during breast tumorigenesis. In preliminary studies of ER+ human breast tumor samples and their matched adjacent normal tissues, a statistically significant decreased relative expression of the exon 3 deleted ER-α mRNA in the tumor compared to the normal breast tissues was noted (Leygue *et al.,* unpublished data).

The available data provide evidence for an extensive and complex pattern of alternative splicing associated with the ER-α gene, which may be altered during breast tumorigenesis.

iib. Specificity of ER-α Splice Variants in Human Breast Tumors. It is unlikely that the mechanisms generating alternatively spliced forms of ER-α are due to a generalized deregulation of splicing processes within breast tumors, since similar variants for the glucocorticoid receptor (16,28), the retinoic acid receptors-α and γ (28) and vitamin D3 receptor (16) have not been found in breast tumor tissues. However, similar splice variants of PR (section 3) were found in both normal and neoplastic breast tissues (31,32).

iic. Expression of ER-α Variant mRNAs During Breast Cancer Progression. As described above the relative expression of at least one ER-α variant mRNA i.e. clone 4 truncated ER-α mRNA, is significantly higher in primary breast tumors with characteristics of poor prognosis (including the presence of concurrent lymph node metastases) compared to primary tumors with good prognostic markers (including lack of concurrent lymph node

metastases) (25). An increased relative expression of exon 5 deleted ER-α mRNA has been found in locoregional breast cancer relapse tissue (in the same breast as the original primary tumor but no lymph node metastases) obtained from patients following a median disease-free interval of 15 months, compared to both the corresponding primary breast tumor (33), and the primary breast tumor tissue of patients who did not relapse during this period. Although the difference did not reach statistical significance, these same authors reported a trend towards higher relative expression of exon 5 deleted ER-α mRNA in primary tumors of women who relapsed compared to primary tumors of those that did not relapse. Together these data suggest that as well as altered expression of ER-α variant mRNA occurring during breast tumorigenesis further changes in ER-α variant expression can occur during breast cancer progression. However, another study has recently found no significant differences in the relative expression of clone 4 truncated, exon 5 deleted, and exon 7 deleted ER-α mRNAs between a series of primary breast tumors and their matched concurrent lymph node metastasis (34), suggesting that altered expression of ER-α variant mRNAs likely occurs prior to the acquisition of the ability to metastasize and therefore may be a marker of future metastatic potential. This hypothesis remains to be tested.

iid. Expression of ER-α variant mRNAs and Endocrine Resistance. The hypothesis that altered forms of ER-α may be a mechanism associated with endocrine resistance has been suggested for some time. Moreover, the identification of ER-α variant mRNAs in human breast biopsy samples (23,35,36) provided good preliminary data for the hypothesis. As well preliminary functional data of the recombinant exon 5 deleted ER-α protein suggested that it possessed constitutive, hormone independent transcriptional activity which was about 15% that of the wild type ER (36). The data using a yeast expression system were also consistent with the correlation of relatively high levels of exon 5 deleted ER-α mRNA in several human breast cancer biopsy samples classified as ER- and PR+ and/or pS2+ (36, 37,38). It was also found that the exon 5 deleted ER-α mRNA was often coexpressed at relatively high levels with the wild type ER-α in many human breast cancers which were ER+ (38). Interestingly, it has been observed that transiently expressed exon 5 deleted ER-α has

an inhibitory effect on endogenously expressed wild type ER-α in MCF-7 human breast cancer cells (39), although it does not decrease the wild type activity to the same extent as hydroxytamoxifen. In contrast, in human osteosarcoma cells exon 5 deleted ER-α was shown to have little effect alone but significantly enhanced estrogen stimulated gene expression by transiently coexpressed wild type ER-α (40). The limitations of transient expression analysis was addressed by two groups who stably overexpressed the exon 5 deleted ER-α in MCF-7 human breast cancer cells (41,42). However, different phenotypes were obtained by the two groups. No effect of the recombinant exon 5 deleted ER-α on growth or estrogen/antiestrogen activity in MCF-7 cells was found in one study (41), while in the other study (42) the overexpression of recombinant exon 5 deleted ER-α in MCF-7 cells was associated with estrogen independent and antiestrogen resistant growth. The reasons for the differences between the two studies are unclear, but may be due to different MCF-7 background or additional changes which could have occurred in the transfectants in addition to transgene expression. It should be noted that the transgene in the Rea and Parker study was episomally maintained while in the study by Fuqua *et al.,* the transgene was presumably integrated into the host chromosomes in a random fashion. The results obtained using recombinant expression of a putative ER-α variant thought to have a functional role in hormonal resistance in human breast cancer, were variable suggesting possibly cell and promoter specific differences. However, irrespective of the function displayed by the exon 5 deleted ER-α under the different experimental conditions, the *in vivo* relevance of the expression levels achieved in the above experiments is likely questionable (24,39).

Several laboratories have developed cell culture models of estrogen independence and antiestrogen resistance. Variable results have been obtained when the association of altered ER-α variant mRNA expression with these phenotypes was investigated. An increased relative expression of an exon 3+4 deleted ER-α variant mRNA was found in an estrogen independent MCF-7 cell line (T5-PRF) derived by long term growth in estrogen depleted medium (43,44). However, this cell line was still sensitive to antiestrogens (43). Although one cell line that was tamoxifen resistant had differential expression of an exon 2 deleted ER-α and

an exon 5 deleted ER-α mRNA compared to the parental cell line (45), other independently derived antiestrogen resistant clones showed no major differences in the expression of ER-α variant mRNAs (46,47).

Investigation of ER-α splice variants using clinical tissue samples has also led to variable conclusions. The relative expression of the clone 4 truncated ER-α variant mRNA was significantly increased in primary breast tumors with characteristics of poor prognosis compared to tumors with good prognostic characteristics (25). As well the relative expression of clone 4 was significantly higher in PR- versus PR+ tumors, suggesting a correlation of increased truncated variant expression and markers of endocrine resistance (25). Furthermore, an increased frequency of detection of ER-α variant mRNAs deleted in exons 2-4 and 3-7 was associated with high tumor grade, but an increased detection of an exon 4 deleted ER-α variant mRNA was associated with low tumor grade (24). The presence of exon 5 deleted ER-α mRNA was found in one study to be associated with increased disease free survival (39). However, no difference in the relative expression of an exon 5 deleted ER-α variant mRNA was found between all tamoxifen resistant tumors and primary control breast tumors (37), although in the subgroup of tamoxifen resistant tumors which were ER+/pS2+, the relative expression of the exon 5 deleted ER-α was significantly greater than the control tamoxifen sensitive group.

Although increased expression of any one ER-α variant does not correlate with tamoxifen resistance of breast cancers overall, its association with and therefore possible involvement in endocrine resistance in some tumors cannot be excluded. Moreover, the presence of multiple types of ER-α variant mRNAs in any one tumor or normal tissue sample has been well documented (24,28) but no data have been published in which total ER-α splice variant expression has been analyzed in relationship to endocrine resistance and prognosis. Although mutations have been found in the ER-α gene in human breast tumors, they are rare and are not more frequent in tamoxifen resistant tumors (48).

iie. Identification of ER-α variant Proteins. An important issue remains the detection of proteins that correspond to those encoded by ER-α variant mRNAs. It is relevant therefore to understand what the structure of these proteins is. The predicted proteins of some of the most frequently detected ER-α variant transcripts are shown schematically in Figure 3. All of these variant transcripts would encode ER-α proteins missing some structural/functional domains of the wild type ER-α. While the ER-α variant transcripts encode several different types of protein, there are some common themes that emerge. A common feature of these putative proteins is the universal presence of the A/B region which is known to contain the cell and promoter specific AF-1 function. Exon 4 deleted and exon 3 + 4 deleted ER-α mRNAs are in frame and encode proteins that do not bind ligand. However, the majority of the most abundantly expressed variant transcripts i.e exon 7 deleted, an exon 4 + 7 deleted and the clone 4 truncated ER-α mRNAs encode proteins which are C-terminally truncated, and cannot bind ligand. So another common feature is the inability to bind ligand. The results obtained in which recombinant techniques were used to measure the function of individual ER-α variants *in vitro* are variable and often depend upon coexpression of the wild type receptor. While it is difficult to make general conclusions many recombinant ER-α variant proteins have been observed to modulate the activity of the wild type receptor. However, the relevance of the relative levels of expression of wild type and variant ER-α proteins that are achieved under the experimental conditions used, is unclear since limited data have been published characterizing the detection of ER-α variant proteins that are encoded by known ER-α variant mRNAs in tissues or cells *in vivo*.

From a different perspective, the prediction that the majority of ER-α variant proteins are C-terminally truncated has implications with respect to the determination of ER status as commonly assayed now. In particular, earlier detection and changes in clinical practice have resulted in smaller amounts of breast tumor tissue being available for assay, and the use of immunohistochemical methods to assess ER status becoming more common. Therefore, depending on the antibodies used, the presence of C-terminally truncated ER-α variant proteins could theoretically influence determination of ER status of the tumor sample. We have

tested this experimentally by transiently transfecting wild type ER-α and clone 4 truncated ER-α expression vectors into Cos-1 cells, and determining ER status of the cells using antibodies either to the N-terminus of the ER-α (Fig 3, 1D5, DAKO) and antibodies to the C-terminus (Fig 3, AER311, Neomarkers). Our preliminary data using different combinations of wild type ER-α and variant ER-α expression vectors transfected into Cos-1 cells indicate that the signals (expressed as H-scores which take into account the intensity of staining and the number of positively staining cells) obtained with the N-terminal and C-terminal antibodies become increasing discrepant (N-terminal>C-terminal signal) with increasing variant expression, presumably due to increased ER-α-like proteins containing the N-terminal region but not the C-terminal region. These preliminary data suggested that increased expression of C-terminally truncated ER-α variant proteins could interfere with the immunohistochemical determination of ER status.

This possibility was investigated in human breast tumor tissues (49). A series of breast tumors was assayed for ER-α using this same set of antibodies, and the H scores from each antibody were compared for each tumor. Interestingly, the tumors fell into two distinct groups, one in which the H-scores obtained with each antibody were consistent and not significantly different from each other, and another group in which the H scores obtained with each antibody were inconsistent and significantly different from each other. Further, in all but one case the H-score was higher for the N-terminal antibody compared to the C-terminal antibody (50). As well, in preliminary experiments using a subset of the original tumor set, we have found similar results using another set of N-terminal and C-terminal ER-α antibodies. Together with the previous experimental data, one interpretation of the tumor data would be that the discrepant tumors had higher levels of C-terminally truncated ER-α–like proteins.

To address the hypothesis that the C-terminally truncated ER-α–like proteins could correspond to proteins encoded by ER-α variant transcripts, we compared expression of ER-α variant mRNAs in the consistent and inconsistent tumors. The results show a significantly higher relative expression and detection of ER-α variant mRNAs which would encode C-terminally truncated proteins in the inconsistent versus the consistent tumors (50). These

results suggest that irrespective of function the expression of significant amounts of C-terminally truncated ER-α variant proteins could interfere with the immunohistochemical determination of ER status which in turn might underlie some of the inconsistencies between ER status and clinical response to endocrine therapy. As well these data would be consistent with the hypothesis that ER-α variant mRNAs may be stably translated *in vivo*. However, such data are indirect and other mechanisms e.g. altered epitope detection, increased proteolytic activity etc, may underlie the discrepant ER-α H-scores found in some human breast tumors. More recently, data published from several independent groups support the detection of ER-α–like proteins in both cell lines and tissues *in vivo* which could correspond to those predicted to be encoded by some previously identified ER-α variant mRNAs. The presence of an exon 5 deleted ER-α protein was demonstrated immunohistochemically in some human breast tumors using a monoclonal antibody specific to the predicted unique C-terminal amino acids of the exon 5 deleted ER-α protein (39). However, although there was a correlation between immunohistochemical detection and presence or absence of exon 5 deleted ER-α mRNA determined by RT-PCR, the group was unable to detect any similar protein by Western blotting, suggesting either very low levels compared to wild type ER-α or differential stability of the variant protein relative to the wild type ER-α during the extraction procedure. In addition, an ER-α-like protein consistent with that predicted to be encoded by the exon 5 deleted ER mRNA is expressed in some BT 20 human breast cancer cell lines as determined by Western blot analysis (51). Western blotting analysis of ovarian tissue has identified both a 65 kDa wild type ER-α protein as well as a 53 kDa protein recognized by ER-α antibodies to epitopes in the N-terminus and C-terminus of the wild type protein, but not with an antibody recognizing an epitope encoded by exon 4 (52). These results correlated with the presence of both wild type and exon 4 deleted ER-α mRNAs in these tissues, and suggested that the 53 kDa protein was derived from the exon 4 deleted ER-α mRNA. More recently, a 61 kDa ER-α-like protein and a more abundant 65 kDa wild type ER-α protein were identified in MCF-7 cells (29). The 61 kDa protein is thought to be encoded by an exon 3 deleted ER-α mRNA expressed at low levels in

these cells, and its comigration both before and after dephosphorylation with the recombinant exon 3 deleted ER-α protein when expressed at higher levels after stable transgene expression in another MCF-7 clone, was thought to strongly suggest its identity with the recombinant exon 3 deleted ER-α protein.

There is accumulating evidence suggesting that variant ER-α proteins which could correspond to those predicted to be encoded by some of the ER-α variant mRNAs can be detected by conventional technologies *in vivo*.

2. Estrogen receptor-β and its Variants.

i.Identification of ER-β mRNA in Human Breast Tissues. With the discovery of a second ER, ER-β, which had similar yet distinct properties to ER-α concerning estrogen and antiestrogen action (10,11,53,54), and could directly interact with the ER-α (55,56), it became important to know if ER-β was expressed in human breast tumors and if so what role it might have in estrogen/antiestrogen action in this tissue.

We have identified the presence of ER-β mRNA both by RT-PCR (12,14) and by RNase protection assay (Figure 4) (14) in some human breast cancer biopsy samples and some human breast cancer cell lines. *In situ* hybridization analysis suggested that expression of ER-β mRNA could be detected in the breast cancer cells of a human breast cancer biopsy sample (14). Using an RT-PCR approach to analyze both ER-β and ER-α mRNA expression in a range of breast tumors (12), the following was observed: a) there was no correlation between ER-β expression and ER-α expression in breast tumors; b) in some cases both ER-β and ER-α mRNA were expressed in the same tumor; c) in those tumors where both ER mRNAs were expressed the relative expression appeared to vary widely amongst tumors. Furthermore, ER-β mRNA can be detected in normal human breast tissues by RT-PCR (13) and RNase protection assay (14). Although there are no data reporting the expression of ER-β protein(s) in human breast tissues as yet, the available information suggest that ER-β may be expressed in both normal and neoplastic human breast tissues and may have a role in these tissues.

ii.Expression of ER-β mRNA during Breast Tumorigenesis. The demonstration of ER–β mRNA expression in both human breast tumors and normal human breast tissue suggests that the well documented role of estrogen in breast tumorigenesis (1,57) may involve both receptors. Using a multiplex RT-PCR approach it has now been shown that the ER-α/ER-β ratio in a small group of ER+ human breast tumors, as assayed by ligand binding, was significantly higher than the ratio in their adjacent normal breast tissues (58). The increase in ER-α/ER-β ratio in breast tumors was primarily due to a significant upregulation of ER-α mRNA expression in all ER+ tumors in conjunction with a lower ER-β mRNA expression in the tumor compared to the normal compartment in some but not all ER+ cases. Interestingly, preliminary data suggest that the level of ER-β mRNA in breast tumors may be correlated with the degree of inflammation (unpublished data). Since *in situ* hybridization data suggest that expression of ER-β mRNA could be detected in the cancer cells of a human breast cancer biopsy sample (14) and that human lymphocytes in lymph nodes can also express ER-β mRNA (14), it is possible that the cell type contributing to the expression of ER-β mRNA may be heterogeneous depending on the tumor characteristics. Irrespective, if the RNA studies reflect the protein levels of the two ERs, the results to date provide evidence to suggest that the role of ER–α and ER-β driven pathways and/or their interaction likely changes during breast tumorigenesis.

iii. Identification of ER-β variant mRNAs in Human Breast Tissues. The presence of multiple ER–α variant mRNAs in both normal and neoplastic human breast tissues has led to the question of the expression of ER-β variant mRNAs. Several ER-β variant mRNAs have now been detected. We have identified an exon 5 + 6 deleted ER-β mRNA in human breast tumors (59). This transcript is in frame and would be expected to encode an ER-β–like protein deleted of 91 amino acids within the hormone binding domain. A human ER-β variant mRNA deleted in exon 5 was identified in MDA MB 231 human breast cancer cells and in some human breast tumor specimens (18). Although this same group was unable to detect an exon 5 deleted ER-β mRNA in normal human breast tissue, we have detected both an exon 5 deleted ER-β mRNA

and an exon 6 deleted ER-β mRNA, as well as an an exon 5 + 6 deleted ER-β mRNA in normal human breast tissue samples (13) and in some human breast tumors. The exon 5 deleted ER-β mRNA and the exon 6 deleted ER-β mRNA are out-of-frame and predicted to encode C-terminally truncated ER-β–like proteins, which would not bind ligand. More recently, several exon 8 deleted human ER-β mRNAs have been identified (17) from a human testis cDNA library and by RT-PCR from the human breast cancer cell line MDA MB 435. These variants have been named human ER-β2-5. It should be noted that human ER-β2 is not the equivalent of the ER-β variant mRNA with an in frame insertion of 69 nucleotides between exons 5 and 6 identified in rodent tissues (13, 60,61) and also named ER-β2. We have been unable to detect an equivalent of the rodent ER-β2 mRNA in any normal or neoplastic human tissue so far studied (13), suggesting species specific differences in alternative splicing of the primary ER-β transcript. Several of the human ER-β variants deleted in exon 8, specifically hER-β2 and hER-β5 can be detected in normal human mammary gland and in several human breast cancer cell lines (17). Interestingly, the predominant type of hER-β exon 8 deleted mRNA present varies amongst the different cell lines. We have confirmed the presence of the hER-β2 and the hER-β5 variant mRNAs in several normal human breast tissue samples from both reduction mammoplasties and normal tissue adjacent to breast tumors (Fig 5, unpublished data). Moreover, we have identified both hER-β2 and the hER-β5 variant mRNAs in several human breast tumor samples (Fig 5, unpublished data). Using a semi-quantitative RT- triple primer PCR approach (26) which simultaneously measures the relative expression of the wild type hER-β1 and the two variants hER-β2 and hER-β5 mRNAs, it appears that in most but not all cases the level of the variant mRNA species exceeds that of the wild type hER-β1 (Fig 5, unpublished data) in both normal and neoplastic human breast tissues. The known sequence of all human ER-β-like transcripts is shown schematically in Figure 6. Also shown in this figure are the proteins predicted to be encoded by these variant hER-β mRNAs. All the hER-β variant mRNAs identified to date are predicted to encode proteins which are altered in the C-terminus

in some fashion, and are unlikely to bind ligand (62). However, published data suggest that some of these variant receptors can homodimerize and bind to ERE *in vitro* , heterodimerize amongst themselves and with wild type hER-β and hER-α (17) and may potentially inhibit preferentially hER-α DNA binding transcriptional activity (62).

iv. Putative Role of ER-β and its Variants in Breast Cancer. Transient transfection studies have provided data which suggest that ER-β1 i.e. the wild type ER-β, can only mediate an antagonist response when bound to tamoxifen-like agents, in contrast to the tamoxifen bound wild type ER-α which can mediate either an antagonist or agonist activity on a basal promoter linked to a classical ERE (53,63). This suggests the possibility that altered relative expression of the two ERs may underlie altered responses to antiestrogens, and could be a mechanism of altered responsiveness to antiestrogens in human breast cancer. As well, the activity of the estrogen bound ER-β1 on AP-1 containing promoters is inhibitory in contrast to that of estrogen bound ER-α, which stimulates transcription (54). Furthermore antiestrogens of all types demonstrated marked transcriptional activity through ER-β1 on promoters that contained AP1 sites (54). Interestingly, a non-ligand binding hER-β variant protein encoded by the variant hER-β2 (also named hER-βcx) can heterodimerize with ER-β1, but preferentially heterodimerizes with ER-α and shows a dominant negative activity only against ER-α mediated transactivation (17,62). It is possible therefore that ER-β1 and its variants could have a direct regulatory role on ER-α activity. Since we have observed an increased ratio of ER-α/ER-β mRNA in human breast tumors compared to their adjacent matched normal tissues, which is primarily due to increased expression of ER-α mRNA in the breast tumor component (58), it is possible that this may translate into unregulated ER-α activity and unregulated growth responses mediated through ER-α which contribute to breast tumorigenesis.

However, there are several issues which have to be addressed before we can begin to develop rational pathophysiologically relevant hypotheses with respect to the role of ER-β and/or its variants in human breast tissues. Firstly, it is not know yet if ER-β and ER-α are

expressed together in the same breast cells or separately in different normal or neoplastic cell populations. Secondly, studies so far have only measured mRNA levels. No studies of protein expression with regard to ER-β-like molecules in human breast have been published to date. Therefore the pathophysiological relevance of the relative levels of ER-β and ER-α expression achieved in transient expression studies, and the resulting functional outcome are unknown. Thirdly, some *in vitro* studies have been done using N-terminally truncated ER-β1 (64), and the functional impact of this is also unknown.

3. Expression of other Steroid Hormone Receptors and their variants in Human Breast Cancer. The observation that the PR gene showed a complex pattern of alternative splicing similar to, although not as extensive as that of ER-α, led to the further characterization of the PR variants (16,31,32). Two commonly expressed variant transcripts identified in human breast tumors and normal human breast tissue were cloned and sequenced. Variant PR mRNAs with either a precise deletion of exon 6 sequences or exon 4 sequences were identified in most breast tumors examined. PR transcripts deleted in exon 2, exons 3+6 or exons 5+6 were also found in a few breast tumors (31,32). The exon 6 deleted transcript was the most abundant and frequently expressed PR variant mRNA in the human breast tumors examined, and specific PCR primers were designed to determine the expression of this transcript relative to the wild type PR using RT-PCR analysis (27). Altered expression of ER-α variant mRNAs was observed previously between normal and neoplastic breast tissue, therefore it was of interest to determine if exon 6 deleted PR mRNA expression was altered during breast tumorigenesis. Using a similar approach to that described previously (27) the relative expression of the exon 6 deleted variant PR mRNA to the wild type PR mRNA was examined in 10 normal reduction mammoplasty samples and 17 breast tumors. The relative expression of the exon 6 deleted PR variant to the wild type PR mRNA was found to be significantly ($P<0.01$) lower in normal breast tissues (median = 4.8%) than in breast tumors (median = 13.9%) (unpublished data).

The exon 2 deleted PR mRNA would encode a C terminally truncated PR-like protein without a DNA or a ligand binding domain (32). The exon 4 deleted PR mRNA is in frame but would encode a protein deleted in exon 4 sequences, missing a nuclear localization signal and the recombinant protein representing exon 4 deleted PR-A did not bind DNA and had little

if any effect on wild type PR-A function (32). Exon 6 deleted PR variant mRNA is out-of-frame and would encode a C-terminally truncated PR-like protein lacking the hormone binding domain, and the exon 5+6 deleted PR variant mRNA is in frame but would encode a protein deleted in exon 5 + 6 sequences of the hormone binding domain (32). Richter *et al.* have demonstrated that recombinant proteins representing the exon 6 deleted PR-A and the exon 5+6 deleted PR-A are dominant negative transcriptional inhibitors of both the wild type PR-A and PR-B (32). It is possible therefore that the presence of PR variant proteins encoded by the identified PR variant mRNAs could modify wild type PR activity and influence responses to endocrine therapies. Interestingly, small variant PR-like proteins have been identified by Western blotting in some breast tumors (32,65,66) which correspond in size to some of the proteins predicted to be encoded by some of the exon deleted PR mRNAs. However, some data suggest that the presence and abundance of PR variant mRNAs may not correlate with the detection of these smaller sized PR immunoreactive species in human breast tumors (66).

The measurement of PR is an important tool in clinical decision making with respect to prognosis and treatment of human breast cancer. Furthermore, the level of PR expression provides important clinical information (67). As the use of enzyme-linked immunosorbent assays (ELISA) and immunohistochemical assays for PR detection increases, it is likely that variant PR expression will interfere with these assays, whatever their function. PR antibodies (AB-52 antibody) used in such assays detect epitopes in the N-terminal region of the wild type molecule shared by truncated PR-like molecules. If all or any of the deleted PR variant mRNAs so far identified are translated into stable proteins, they will be co-detected with the wild type PR in such assays. Presence of PR variants might also be a factor contributing to discrepancies between biochemical measurement and immunological detection of PR. Indeed the potential for ER-α variant expression to interfere with the immunohistochemical assessment of ER status has been documented (49,50,68).

4. Conclusions and Controversies.

The multifaceted nature of the ER is suggested by the expression of ER-α mRNA, ER-β mRNA and their variant mRNAs in both normal and neoplastic human breast tissues (Figure 7). There is a large body of molecular data which support at least the potential for this multifaceted nature of the ER and therefore estrogen/antiestrogen signalling in both normal

and neoplastic human breast tissues. Alterations in the relative expression of several ER-like mRNAs have been shown to occur during breast tumorigenesis and the relative frequency of detection and relative expression of individual ER-like mRNAs can be correlated with different prognostic characteristics in breast cancer. This in turn suggests a possible role in breast tumorigenesis and possibly hormonal progression in breast cancer. However, there are still major gaps that need to be addressed before we have a clear idea of the pathophysiological and functional relevance of the experimental results so far in hand. Unequivocal data are required to support the *in vivo* detection of variant ER-α, variant ER-β and wild type ER-β proteins which correspond to the variant ER-α, variant ER-β and wild type ER-β mRNA species, respectively. There is a need to experimentally determine putative function using expression levels which reflect pathophysiological levels of expression. There is a need to know if the two wild type ER receptors and/or their variants are co-expressed in the same cells within the heterogeneous normal and neoplastic breast tissues. Further, given the detection of multiple forms of variant ER-like species in any one breast tissue sample the limitations in interpreting data from experimental systems in which only one variant species is considered in the presence or absence of wild type protein needs to be understood.

Acknowledgements. This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). LCM is a Medical Research Council of Canada (MRC) Scientist, PHW is a MRC Clinician-Scientist, EL is a recipient of a USAMRMC Postdoctoral Fellowship, AC is a recipient of a Manitoba Health Research Council (MHRC) Studentship.

Figure Legends.

Figure 1.

Top panel. Schematic representation of wild type ER-α (WT-ER) cDNA and primers allowing co-amplification of most of the described exon-deleted ER-α variants: ER-α cDNA contains 8 different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (AF-2). 1/8U and 1/8L primers allow amplification of 1381 bp fragment corresponding to wild type ER-α mRNA. Co-amplification of all possible exon-deleted or inserted variants that contain exon 1 and 8 sequences can occur. Amplification of the previously described ER-α variant mRNAs deleted in exon 3 (D3-ER), exon 4 (D4-ER), exon 7 (D7-ER), both exons 3 and 4 (D3-4-ER), exons 2 and 3 (D2-3-ER), exons 4 and exon 7 (D4/7-ER), would generate 1264 bp, 1045 bp, 1197 bp, 928 bp, 1073 bp and 861 bp fragments, respectively.

Bottom panel. Co-amplification of wild type ER-α and deleted variant mRNAs in breast tumor samples: Total RNA extracted from ER positive (+) and ER negative (-) breast tumors was reverse transcribed and PCR amplified as described (24) using 1/8 U and 1/8L primers. Radioactive PCR products were separated on a 3.5% acrylamide gel and visualized by autoradiography. Bands reproducibly obtained within the set of tumors studied and that migrated at 1381 bp, 1197 bp, 1045 bp, 928 bp, 889 bp, 861 bp, 737 bp and 580 bp were identified as corresponding to WT-ER mRNA and variant mRNAs deleted in exon 7 (D7-ER), exon 4 (D4-ER), both exons 3 and 4 (D3-4-ER), exons 2, 3 and 7 (D2-3/7-ER), both exons 4 and 7 (D4/7-ER), exons 2, 3 and 4 (D2-3-4-ER), and within exon 3 to within exon 7 (D-3-7-ER), respectively. PCR products indicated by dashes (-), barely detectable within the tumor population, i.e present in less than or equal to 3 particular tumors, have not yet been identified. M: Molecular weight marker (phi174, Gibco BRL, Grand Island, NY). Adapted from (24).

Figure 2.

Linear regression analysis of the relationship between the clone 4 truncated ER-α mRNA and the wild type ER-α mRNA in the various groups. Closed circles represent the "good" prognosis/ER+PgR+ group; open circles represent the "good" prognosis/ER+PgR- group;

closed squares represent the "poor" prognosis/ ER+PgR+ group; open squares represent the "poor" prognosis /ER+PgR- group. Good vs Poor, p = 0.0004; PgR- vs PgR+. P = 0.011. Reproduced from (25).

Figure 3.

Schematic representation of the ER-α variant proteins predicted to be encoded by ER-α variant mRNAs. Identical sequence is depicted by numbered exons. U = amino acid sequence unrelated to wild type human estrogen receptor-α amino acid sequence. U sequences are unique to any particular variant. The position of N- and C-terminal epitopes recognized by 1D5 and AER311 antibodies, respectively, are indicated.

Figure 4.

Detection of ERβ mRNA in human breast tumors by RNase protection assay. A. Schematic representation of hERβ mRNA showing various exon sequences and identifying the riboprobe position and size of the expected protected fragment (259 bp). Total RNA was isolated from 7 breast tumor samples and 25 μg were used in an RNase protection assay as previously described (21). Ovarian RNA (ovary) was used as a positive control.

Figure 5.

RT-triple primer PCR analysis (26) of the relative expression of human estrogen receptor-β1 (ER-β1), human estrogen receptor-β5 (ER-β5) and human estrogen receptor- β2 (ER-β2) mRNAs in normal (N) and breast tumor (T) tissue samples.

Figure 6.

Human ER-β isoforms. All hER-β isoforms are aligned. White boxes indicate identity of amino acid between sequences. Amino acid positions of the different structural domains are indicated for the hER-β1 short (14) that contains 8 extra N-terminal amino acids compared to the first hER-β described (10). hER-β1 long (Genbank AF051427) contains 45 additional N-terminal amino acids. hER-β1Δ5 (13,18), hER-β1Δ6 (13), hER-β2 (Genbank AF051428, AB006589cx), hER-β3 (Genbank AF060555), hER-β4 (Genbank AF061054), hER-β5 (Genbank AF061055) are truncated and contain different C-terminal amino acids (black boxes). hER-β Δ5-6 (13)(Genbank AF074599) is missing 91 amino acids within the LBD/AF2

domain. For each receptor, the length (aa) and the calculated molecular mass (kDa) when known or corresponding to the short (S) or the long (L) forms of the putative proteins are given. Broken boxes and question marks indicate that flanking amino acid sequences are unknown.

Figure 7.

Schematic representation of the known and unknown (?) multiple facets of the estrogen receptor (R).

References.

1. **Vorherr H** 1980 Breast Cancer. Urban & Schwarzenberg Press, Baltimore.

2. **Clarke R, Skaar T, Baumann K, Leonessa F, James M, Lippman M, Thompson EW, Freter C Brunner N** 1994 Hormonal carcinogenesis in breast cancer: cellular and molecular studies of malignant progression. Breast Cancer Res Treat 31, 237-248.

3. **Green S, Kumar V, Krust A, Walter P Chambon P** 1986 Structural and functional domains of the estrogen receptor. Cold Spring Harb Symp Quant Biol 51 Pt 2, 751-758.

4. **Tsai MJ O'Malley BW** 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily. Annu Rev Biochem **63**, 451-486.

5. **Horwitz KB, McGuire WL, Pearson OH Segaloff A** 1975 Predicting response to endocrine therapy in human breast cancer: a hypothesis. Science 189, 726-727.

6. **Horwitz K, Koseki Y McGuire W** 1978 Estrogen control of progesterone receptor in human breast cancer: role of estradiol and antiestrogen. Endocrinology 103, 1742-1751.

7. **Murphy L** 1995 Mechanisms associated with the progression of breast cancer from hormone dependence to independence. The Pezcoller Foundation Journal 2, 6-16.

8. **Taylor R, Powles T, Humphreys J, Bettelheim R, Dowsett M, Casey A, Neville A, Coombes R** 1982 Effects of endocrine therapy on steroid-receptor content of breast cancer. Br J Cancer 45, 80-84.

9. **Katzenellenbogen B** 1991 Antiestrogen resistance: mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. J Natl Cancer Inst 83, 1434-1435.

10. **Mosselman S, Polman J Dijkema R** 1996 ER-beta: identification and characterization of a novel human estrogen receptor. FEBS Letters 392, 49-53.

11. **Kuiper G, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A** 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci, U.S.A. 93, 5925-5930.

12. **Dotzlaw H, Leygue E, Watson P Murphy L** 1997 Expression of estrogen receptor-beta in human breast tumors. J Clin Endocrinol Metabol 82, 2371-2374.

13. **Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC, Watson PH** 1998 Estrogen receptor-beta mRNA variants in human and murine tissues. Mol Cell Endocrinol 138, 199-203.

14. **Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M Gustafsson J-A** 1997 Human estrogen receptor β gene structure, chromosomal localization and expression pattern. J Clin Endocrinol Metabol 82, 4258-4265.

15. **Murphy L, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson P** 1997 Oestrogen receptor variants and mutations in human breast cancer. Ann Med 29, 221-234.

16. **Murphy L, Dotzlaw H, Leygue E, Coutts A, Watson P** 1998 The Pathophysiological role of estrogen receptor variants in human breast cancer. J Steroid Biochem Mol Biol 65, 175-180.

17. **Moore J, McKee D, Slentz-Kesler K, Moore L, Jones S, Horne E, Su J-L, Kliewer S, Lehmann J, Willson T** 1998 Cloning and characterization of human estrogen receptor β isoforms. Biochem Biophys Res Commun 247, 75-78.

18. **Vladusic E, Hornby A, Guerra-Vladusic F, Lupu R** 1998 Expression of estrogen receptor β messenger RNA variant in breast cancer. Cancer Res 58, 210-214.

19. **Murphy L, Dotzlaw H, Leygue E, Douglas D, Coutts A, Watson P** 1997 Estrogen receptor variants and mutations: a review. J Steroid Biochem Mol Biol 62, 363-372.

20. **McGuire WL, Chamness GC, Fuqua SA** 1991 Estrogen receptor variants in clinical breast cancer. Mol Endocrinol 5, 1571-1577.

21. **Dotzlaw H, Alkhalaf M, Murphy LC** 1992 Characterization of estrogen receptor variant mRNAs from human breast cancers. Mol Endocrinol 6, 773-785.

22. **Fuqua SA, Allred DC, Auchus RJ** 1993 Expression of estrogen receptor variants. J Cell Biochem Suppl 17G, 194-197.

23. **Murphy LC, Dotzlaw H** 1989 Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. Mol Endocrinol 3, 687-693.

24. **Leygue E, Huang A, Murphy L, Watson P** 1996 Prevalence of estrogen receptor variant messenger RNAs in human breast cancer. Cancer Res 56, 4324-4327.

25. **Murphy LC, Hilsenbeck SG, Dotzlaw H Fuqua SAW** 1995 Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. Clin Cancer Res 1, 155-159.

26. **Leygue E, Murphy L, Kuttenn F, Watson P** 1996 Triple primer polymerase chain reaction. A new way to quantify truncated mRNA expression. Am J Pathol 148, 1097-1103.

27. **Leygue ER, Watson PH, Murphy LC** 1996 Estrogen receptor variants in normal human mammary tissue. J Natl Cancer Inst 88, 284-290.

28. **Pfeffer U, Fecarotta E, Vidali G** 1995 Coexpression of multiple estrogen receptor variant messenger RNAs in normal and neoplastic breast tissues and in MCF-7 cells. Cancer Res 55, 2158-2165.

29. **Erenburg I, Schachter B, Lopez RM, Ossowski L** 1997 Loss of an estrogen receptor isoform (ER-alpha deleted 3) in breast cancer and the consequences of its reexpression: interference with estrogen-stimulated properties of malignant transformation. Mol Endocrinol 11, 2004-2015.

30. **Wang Y, Miksicek RJ** 1991 Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol 5, 1707-1715.

31. **Leygue E, Dotzlaw H, Watson PH, Murphy LC** 1996 Identification of novel exon-deleted progesterone receptor variant mRNAs in human breast tissue. Biochem Biophys Res Commun 228, 63-68.

32. **Richter J, Lange C, Wierman A, Brooks K, Tung L, Takimoto G, Horwitz KB** 1998 Progesterone receptor variants found in breast cells repress transcription by wild type receptors. Breast Cancer Res Treat 48, 231-241.

33. **Gallacchi P, Schoumacher F, Eppenberger-Castori S, Landenberg EV, Kueng W, Eppenberger U, Mueller H** 1998 Increased expression of estrogen receptor exon 5 deletion variant in relapse tissues of human breast cancer. Int J Cancer 79, 44-48.

34. **Leygue E, Hall R, Dotzlaw H, Watson P, Murphy L** 1998 Estrogen receptor-alpha variant mRNA expression in primary human breast tumors and matched lymph node metastases. Br J Cancer, in press.

35. **Garcia T, Lehrer S, Bloomer WD, Schachter B** 1988 A variant estrogen receptor messenger ribonucleic acid is associated with reduced levels of estrogen binding in human mammary tumors. Mol Endocrinol 2, 785-791.

36. **Fuqua SA, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Malley BW, McGuire WL** 1991 Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 51, 105-109.

37. **Daffada AA, Johnston SR, Smith IE, Detre S, King N, Dowsett M** 1995 Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen

resistance and progesterone receptor/pS2 status in human breast cancer. Cancer Res 55, 288-293.

38. **Zhang QX, Borg A Fuqua SA** 1993 An exon 5 deletion variant of the estrogen receptor frequently coexpressed with wild type estrogen receptor in human breast cancer. Cancer Res 53, 5882-5884.

39. **Desai A, Luqmani Y, Coope R, Dagg B, Gomm J, Pace P, Rees C, Thirunavukkarasu V, Shousha S, Groome N, Coombes R, Ali S** 1997 Presence of exon 5 deleted oestrogen receptor in human breast cancer: functional analysis and clinical significance. Br J Cancer 75, 1173-1184.

40. **Chaidarun S Alexander J** 1998 A tumor specific truncated estrogen receptor splice variant enhances estrogen stimulated gene expression. Mol Endocrinol 12, 1355-1366.

41. **Rea D Parker MG** 1996 Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. Cancer Res 56, 1556-1563.

42. **Fuqua SA Wolf DM** 1995 Molecular aspects of estrogen receptor variants in breast cancer. Breast Cancer Res Treat 35, 233-241.

43. **Coutts A, Davie J, Dotzlaw H, Murphy L** 1996 Estrogen regulation of nuclear matrix-intermediate filament proteins in human breast cancer cells. J Cell Biochem 63, 174-184.

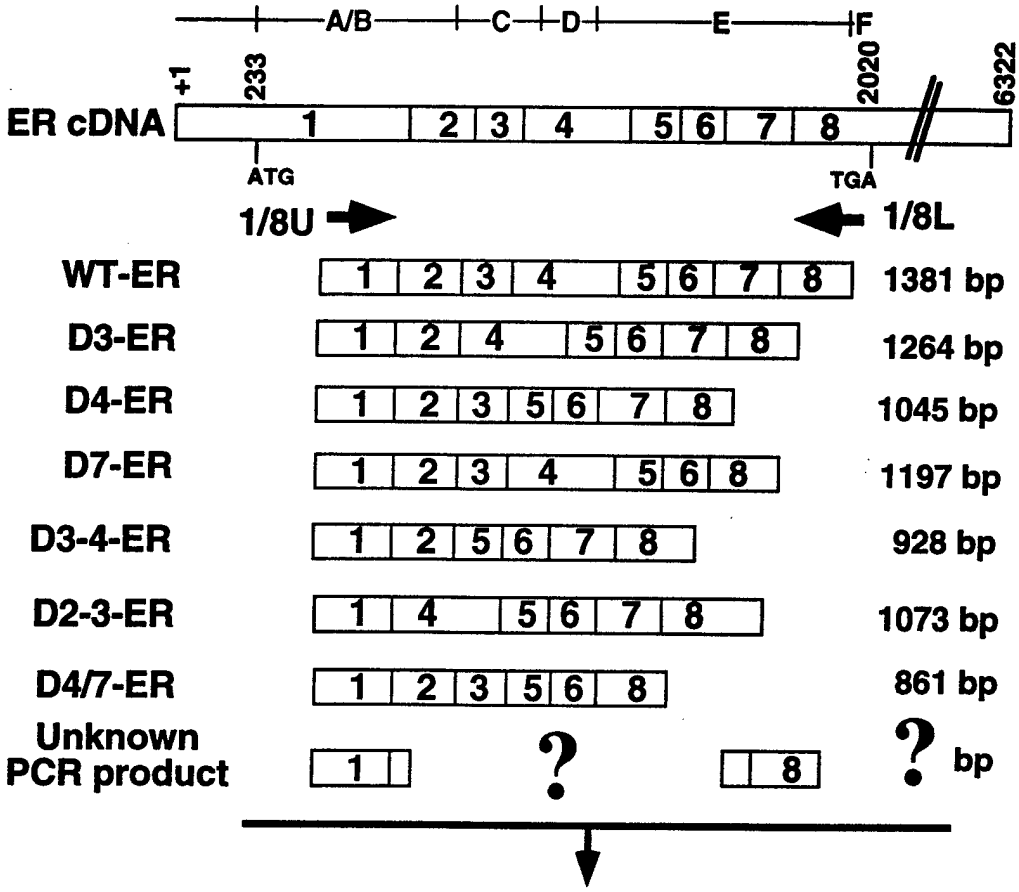
44. **Coutts A, Leygue E, Murphy L** 1997 Mechanisms of hormone independence in human breast cancer cells. Abstract 1998. In: 88th Annual Meeting of the American Association for Cancer Research pp. 296, San Diego, California.

45. **Madsen MW, Reiter BE Lykkesfeldt AE** 1995 Differential expression of estrogen receptor mRNA splice variants in the tamoxifen resistant human breast cancer cell line, MCF-7/TAMR-1 compared to the parental MCF-7 cell line. Mol Cell Endocrinol 109, 197-207.

46. **Madsen MW, Reiter BE, Larsen SS, Briand P, Lykkesfeldt AE** 1997 Estrogen receptor messenger RNA splice variants are not involved in antiestrogen resistance in sublines of MCF-7 human breast cancer cells. Cancer Res 57, 585-589.

47. **Brunner N, Boysen B, Jirus S, Skaar T, Holst-Hanson C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua S, Clarke R** 1997 MCF7/LCC9: an antiestrogen resistant MCF 7 variant in which acquired resistance to the steroidal antiestrogen ICI 182780 confers an early cross resistance to the nonsteroidal antiestrogen tamoxifen. Cancer Res 57, 3486-3493.

Figure 1



PCR Co-amplification of WT-ER and all known and unknown deleted-ER variant mRNAs

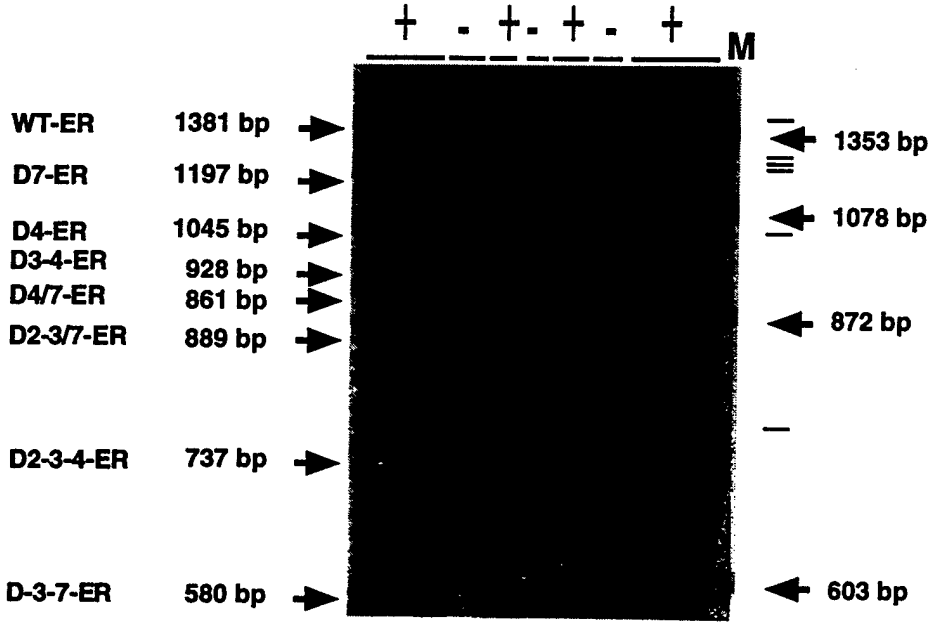
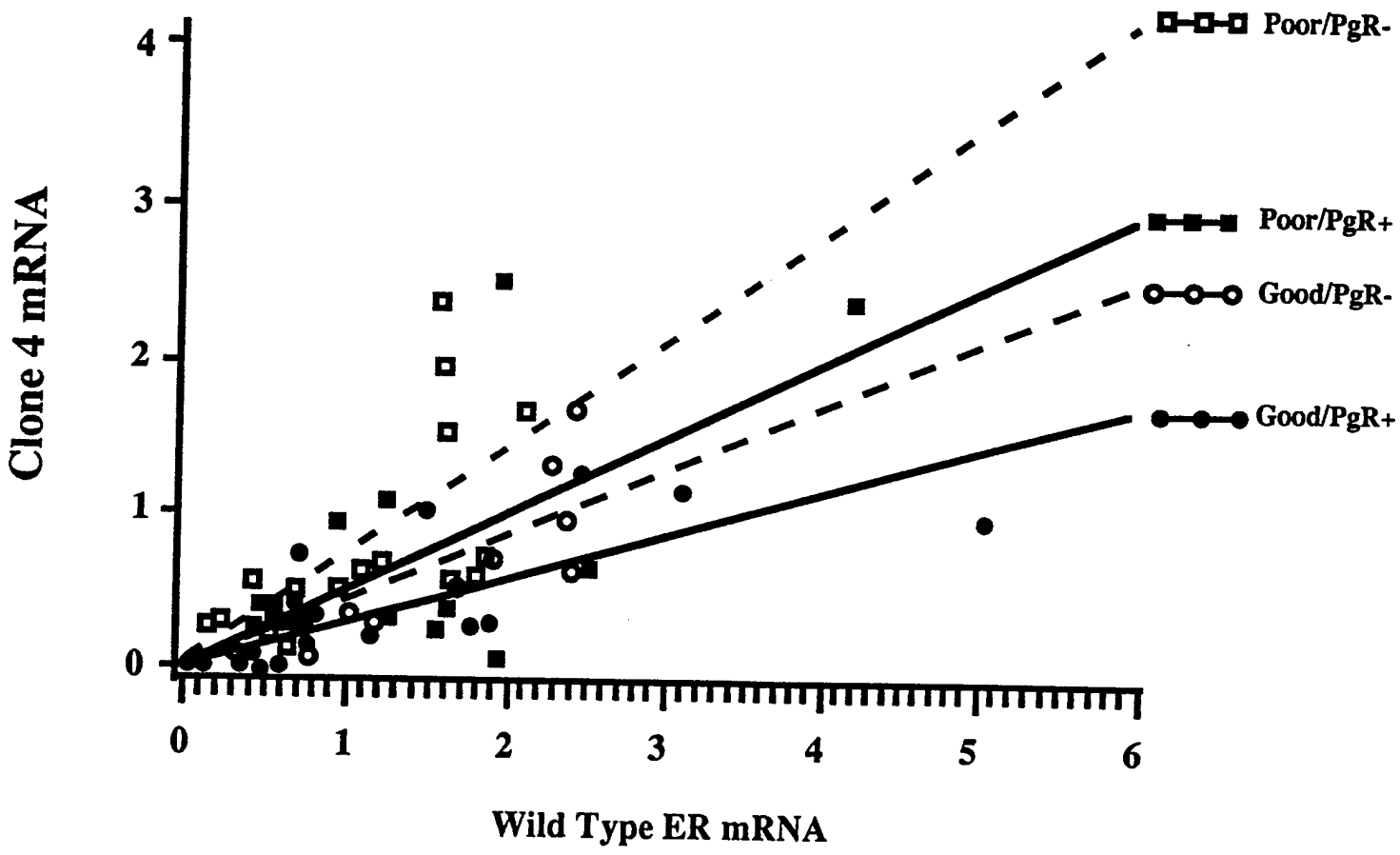
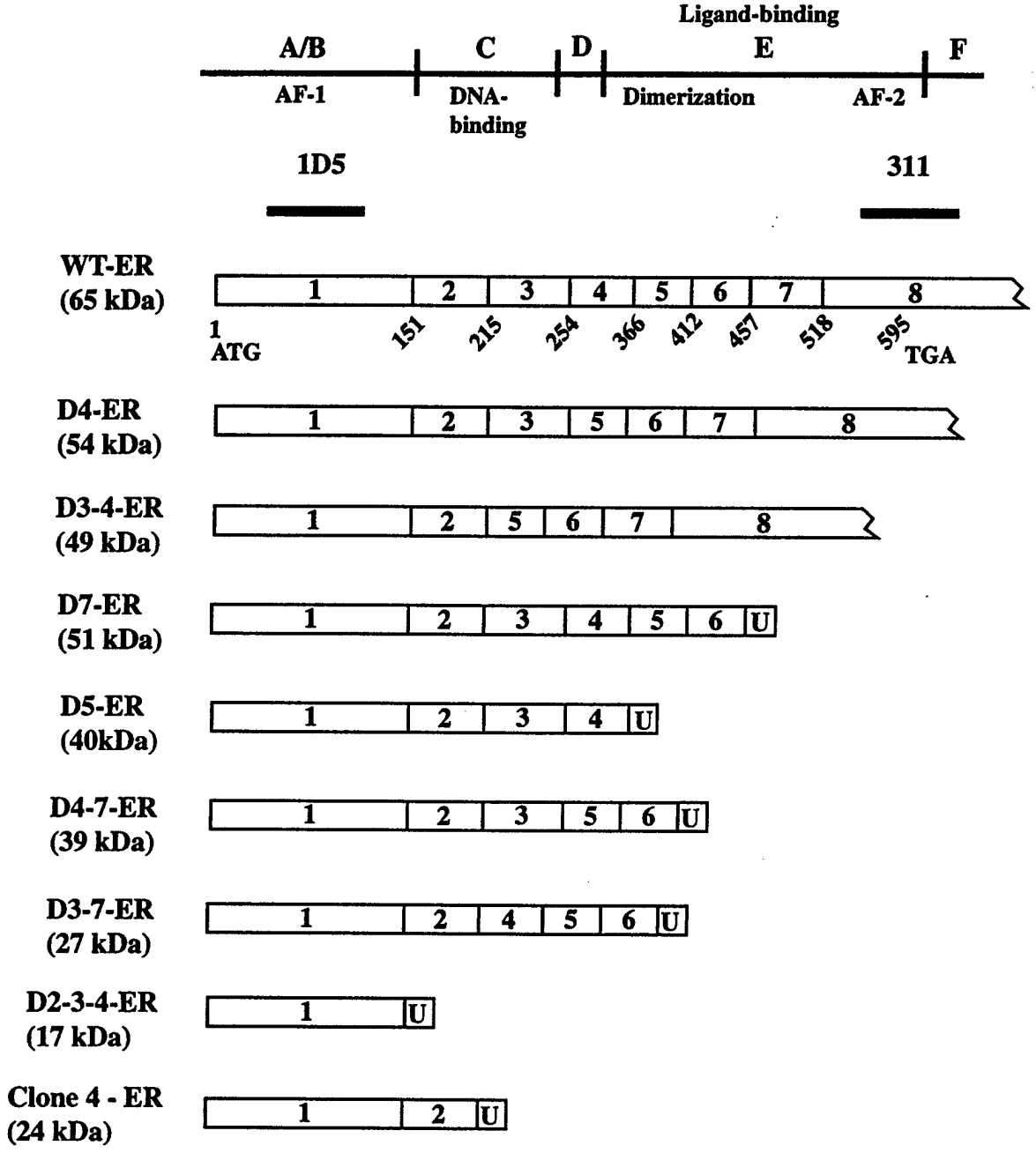
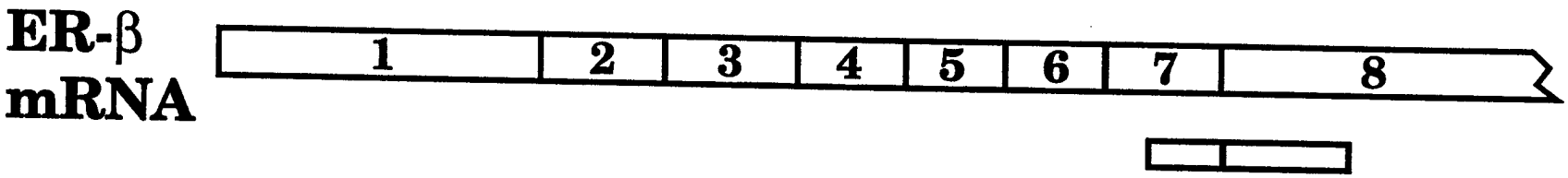


FIGURE 2.

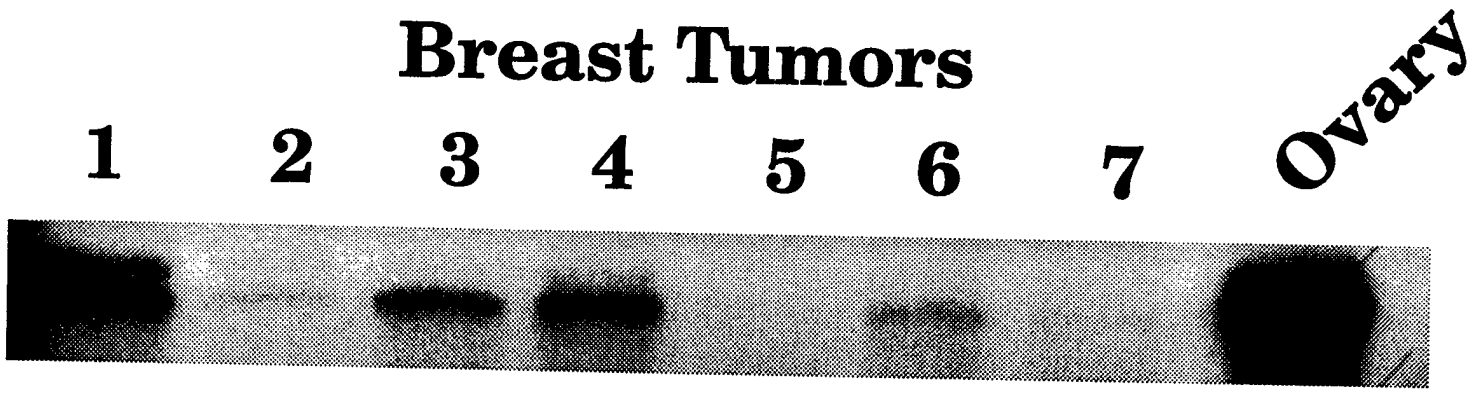

Clone 4 mRNA
Wild Type ER mRNA
0
1
2
3
4
5
6
Poor/PgR-
Poor/PgR+
Good/PgR-
Good/PgR+

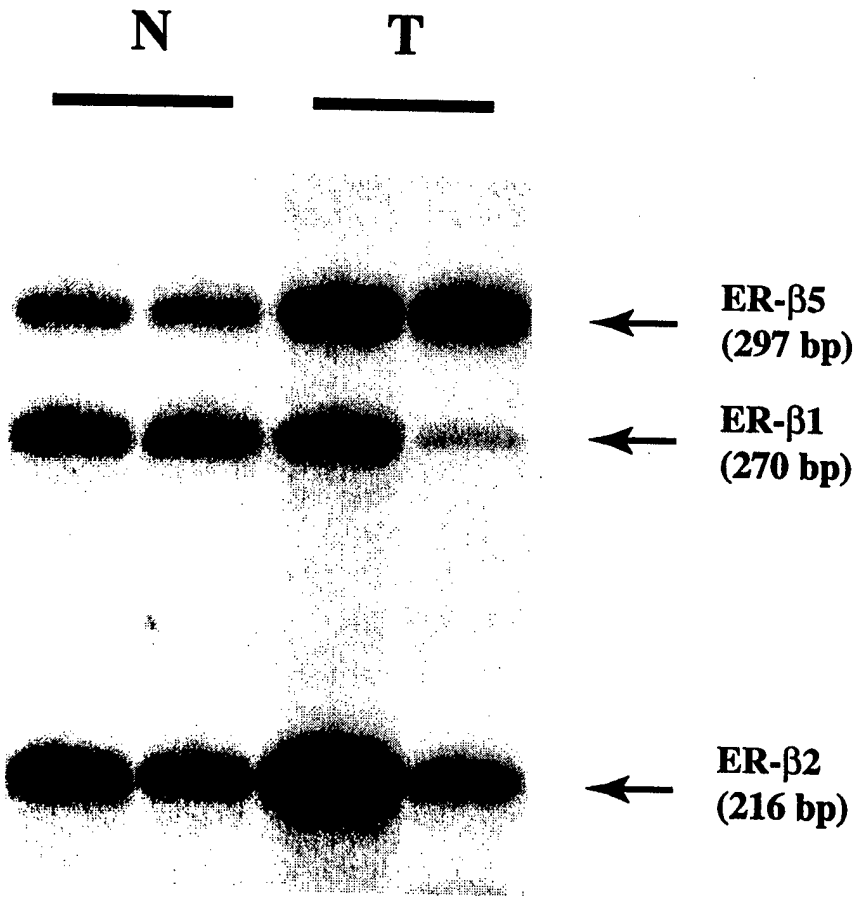

Ligand-binding
A/B
C
D
E
F
AF-1
DNA-binding
Dimerization
AF-2
1D5
311
WT-ER (65 kDa)
1
2
3
4
5
6
7
8
1 ATG
151
215
254
366
412
457
518
595
TGA
D4-ER (54 kDa)
D3-4-ER (49 kDa)
D7-ER (51 kDa)
U
D5-ER (40kDa)
D4-7-ER (39 kDa)
D3-7-ER (27 kDa)
D2-3-4-ER (17 kDa)
Clone 4 - ER (24 kDa)

A.


ER-β
mRNA
1
2
3
4
5
6
7
8
Riboprobe - 259 bp

B.


Breast Tumors
1
2
3
4
5
6
7
Ovary


N
T
ER-β5
(297 bp)
ER-β1
(270 bp)
ER-β2
(216 bp)

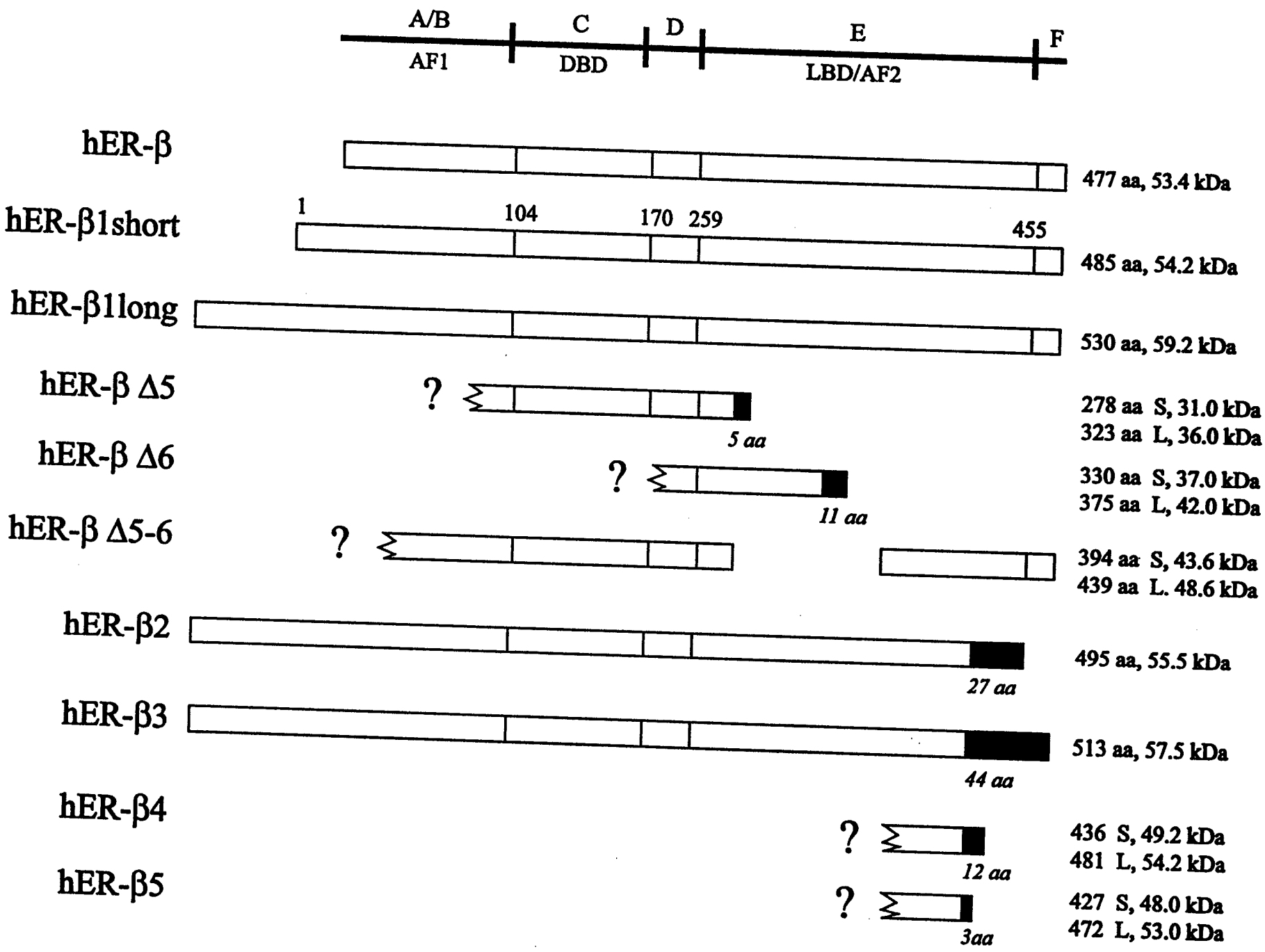
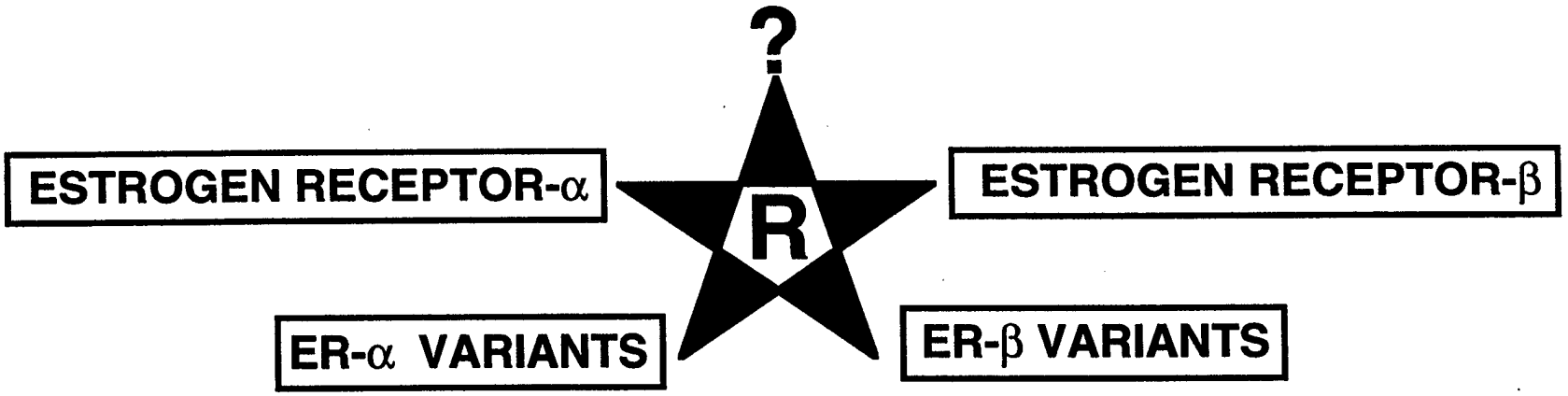

A/B
AF1
C
DBD
D
E
LBD/AF2
F
hER-β
477 aa, 53.4 kDa
hER-β1short
1
104
170
259
455
485 aa, 54.2 kDa
hER-β1long
530 aa, 59.2 kDa
hER-β Δ5
?
5 aa
278 aa S, 31.0 kDa
323 aa L, 36.0 kDa
hER-β Δ6
?
11 aa
330 aa S, 37.0 kDa
375 aa L, 42.0 kDa
hER-β Δ5-6
?
394 aa S, 43.6 kDa
439 aa L. 48.6 kDa
hER-β2
27 aa
495 aa, 55.5 kDa
hER-β3
44 aa
513 aa, 57.5 kDa
hER-β4
?
12 aa
436 S, 49.2 kDa
481 L, 54.2 kDa
hER-β5
?
3aa
427 S, 48.0 kDa
472 L, 53.0 kDa

Figure 7.


?
ESTROGEN RECEPTOR-α
ESTROGEN RECEPTOR-β
R
ER-α VARIANTS
ER-β VARIANTS

APPENDIX 7

[CANCER RESEARCH 58, 4071–4074, September 15, 1998]

Advances in Brief

Elevated Mitogen-activated Protein Kinase Activity in Estrogen-nonresponsive Human Breast Cancer Cells[1]

Amanda S. Coutts and Leigh C. Murphy[2]

Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, R3E 0W3 Canada

Abstract

The mitogen-activated protein kinase (MAPK) signal transduction pathway plays an essential role in cell cycle progression and can be activated by many growth factor/mitogen pathways including estrogen. MAPK has also been implicated in ligand-independent activation of estrogen receptor-α (ER-α). The development of estrogen-independent growth in breast cancer is likely a first step in progression to hormone independence and antiestrogen resistance. We examined MAPK expression and activity in T5-PRF and T5 human breast cancer cells. T5-PRF is an estrogen-nonresponsive cell line developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. MAPK activity measured *in vitro* was significantly higher ($P < 0.05$) in T5-PRF compared with T5 cells. Western blot analyses showed increased levels of active dually phosphorylated MAPK in T5-PRF cell extracts compared with T5. The increased activity and expression of MAPK may contribute to the estrogen nonresponsive growth phenotype and ligand-independent activity of ER in T5-PRF cells.

Introduction

MAPKs[3] (or ERKs) are a family of protein kinases involved in transmitting signals from a variety of stimuli from the cell membrane to the nucleus (1). The MAPKs, ERK1 and ERK2, are activated by mitogenic stimuli from growth factor receptors such as epidermal growth factor receptor (1). Once activated, ERK1 and ERK2 phosphorylate a variety of proteins, including transcription factors, to effect changes in gene expression. A cascade of protein kinases regulate and activate MAPK via phosphorylation on both threonine and tyrosine residues (1).

Several studies have suggested a potential role for the MAPK signaling pathway in the initiation and pathogenesis of breast cancer. MAPK activity has been shown to be elevated in primary breast cancer compared with benign breast tissue and has also been shown to be overexpressed in metastatic cells within lymph nodes of breast cancer patients (2). Constitutive expression of Raf-1 kinase (an upstream activator of the MAPK pathway) in MCF-7 human breast cancer cells resulted in estrogen-independent growth (3). ER-α, like other members of the steroid-thyroid hormone receptor superfamily, is functionally regulated via phosphorylation by several protein kinases including MAPK (4–8). Phosphorylation is believed to play a role in regulating many aspects of steroid hormone receptor function including DNA binding and transcriptional activation. Ser118 of the ER-α has been shown to be phosphorylated by MAPK in response to receptor activation by growth factors including epidermal growth factor, and mutation of this site to an alanine residue severely diminished ER-α transcriptional ability (4, 7).

Phosphorylation of ER-α by MAPK has been implicated in hormone-independent activation of the ER-α by steroid-independent activators of ER-α (4, 7). The development of estrogen-independent growth in human breast cancer is thought to be one of the initial steps in the progression to hormone independence and resistance to endocrine therapies. However, the mechanisms responsible for the development of estrogen independence and the presence of continued expression of ER-α are poorly understood. To address this, we have developed a breast cancer cell model of apparent estrogen independence (9). T5 human breast cancer cells are ER-α positive, and estrogen treatment in culture results in increased proliferation of these cells. An estrogen-nonresponsive cell line, T5-PRF, was developed from T5 cells by chronically depleting the cells of estrogen in long-term culture. These cells are insensitive to the growth-stimulatory effects of estrogen seen in the parent cell line while still retaining expression of the ER-α (9). We have characterized and compared MAPK activity and levels in these two cell lines to determine whether differences in MAPK activity could contribute to the estrogen-independent phenotype of the T5-PRF cell line.

Materials and Methods

Reagents. Monoclonal mouse anti-phospho-MAPK antibody (#9105S) was from New England Biolabs (Beverly, MA). Polyclonal rabbit anti-ERK1 (C-16) was from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). [^{32}P]γATP was purchased from ICN (St-Laurent, Quebec, Canada). Estradiol-17β was from Sigma Chemical Co. (St. Louis, MO). PD 98059 was purchased from Calbiochem (La Jolla, CA.). DMEM powder, fetal bovine serum, and myelin basic protein were purchased from Life Technologies, Inc. (Burlington, Ontario, Canada). All other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario, Canada). Cyclic AMP-dependent protein kinase inhibitor peptide was purchased from Bachem (Torrance, CA). ICI 164,384 was a gift from ICI (Macclesfield, Cheshire, United Kingdom). [^{14}C]Chloramphenicol was obtained from NEN (Lachire, Quebec, Canada).

Cell Culture. T5 human breast cancer cells were routinely cultured in DMEM containing 5% v/v FCS, 1% w/v glucose, 2 mM glutamine, and 100 units of penicillin-streptomycin as described previously (9). T5-PRF cells were routinely cultured in PRF/DMEM supplemented with 5% v/v twice charcoal dextran-stripped FCS and 1% w/v glucose, 2 mM glutamine, and 100 units of penicillin-streptomycin as described previously (9).

MAPK Assay. MAPK activity *in vitro* was measured using myelin basic protein as a substrate as described previously (10). For experiments performed in serum-free conditions, cells were plated in PRF/DMEM, and the following day the medium was changed to PRF/DMEM minus serum and changed every day for 7 days. To measure basal activity, T5 cells were passaged once in PRF/DMEM, and both T5 and T5-PRF cells were set up in PRF/DMEM in 100-mm dishes at ~0.2×10^6 cells and harvested in MAPK buffer [100 mM β-glycerophosphate, 1 mM sodium orthovanadate (pH 10), 2 mM EGTA, 20 mM Tris-HCl (pH 7.4), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM 4-[2-aminoethyl]benzenesulfonyl fluoride hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 0.2 mM benzamidine] 3 days later. Extracts

Received 6/29/98; accepted 7/27/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the United States Army Medical Research and Materiel Command, the Medical Research Council of Canada, and the Manitoba Health Research Council. L. C. M. is a Medical Research Council of Canada Scientist, and A. S. C. holds a Manitoba Health Research Council Studentship.

[2] To whom requests for reprints should be addressed. Phone: (204) 789-3233; Fax: (204) 789-3900; E-mail: lcmurph@cc.umanitoba.ca.

[3] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; ER, estrogen receptor; PRF, phenol red free; CAT, chloramphenicol acetyltransferase; MEK, MAPK kinase.

for MAPK protein detection were obtained in a similar fashion. For experiments using PD 98059, cells were treated an hour before harvesting with 50 μM inhibitor.

Western Blotting and Immune Detection. Extracts obtained for MAPK assay were analyzed using 7.5% SDS-PAGE with a 4% stacking gel at 200 V for 45 min at room temperature according to the Laemmli method (11). Gels were transferred to nitrocellulose using 3-[cyclohexylamino]-1-propanesulfonic acid transfer buffer [10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11), 20% methanol] and transferred for 1 h at 120 V at 4°C. Blots were blocked overnight in 5% skimmed milk/Tris-buffered saline containing 0.5% Tween-20 (TBS-T). Blots were incubated with mouse anti-phospho-MAPK antibody for detection of dually phosphorylated MAPK (NEB, Beverly, MA; 1:1000 in 1% skimmed milk/TBS-T) or rabbit anti-ERK1 (C-16) for detection of total MAPK protein (Santa Cruz Biotechnology; 1:1000 in 1% skimmed milk/TBS-T) for 4 h at room temperature. Blots were incubated with the appropriate secondary antibody (anti-mouse, Jackson ImmunoResearch Laboratories, West Grove, PA; anti-rabbit, Sigma, St. Louis, MO) for 1 h at room temperature, 1:1000 in 1% skimmed milk/TBS-T. Detection was carried out using the ECL detection system according to the manufacturer's instructions (Amersham, Buckinghamshire, United Kingdom).

Transient Transfections. T5 cells were passaged once in PRF-DMEM, and T5 and T5-PRF cells were set up in PRF-DMEM at 1–2 $\times$ 10^6 cells/100-mm dish the day before transfection. Cells were transfected with 2 μg of ERE-tk-CAT (12) and 5 μg of pCH110 (β-galactosidase expression vector; Pharmacia) overnight using the calcium phosphate/glycerol shock method using an equal volume of 2$\times$ BBS buffer (50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, and 1.5 mM Na_2HPO_4, pH 6.95). The following day, the cells were subjected to a 2-min glycerol shock (20% v/v), were washed twice with 1$\times$ PBS, and given fresh medium plus or minus 10 nM estradiol-17β, plus or minus 50 μM PD 98059 (MEK inhibitor; Calbiochem). Two h later, PD 98059-treated dishes were given a second dose of 50 μM PD 98059, and 18 h later, dishes were harvested and cell extracts were obtained. Cell extracts were assayed for β-galactosidase activity to control for transfection efficiency, and CAT assays were performed as described previously (13).

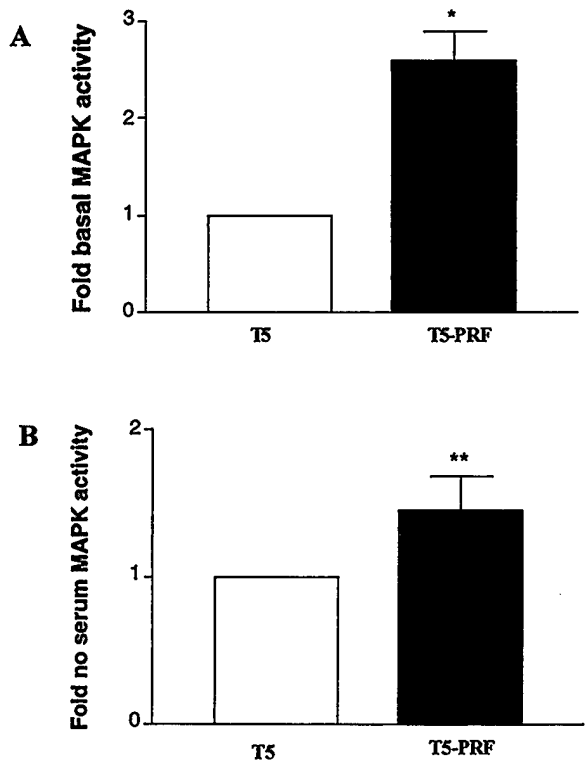


Fig. 1. MAPK activity *in vitro*. Cell extracts for measurement of *in vitro* MAPK activity were obtained as described in "Materials and Methods." Myelin basic protein was used as a substrate to measure kinase activity, and the assay was allowed to proceed for 10 min at 30°C . Histograms represent fold difference in MAPK activity after setting the activity of T5 parent cells to 1.0. *A*, basal activity: fold increase is 2.6 $\pm$ 0.3, $n = 3$. *, $P < 0.05$, Student's *t* test. *Bars*, SE. *B*, serum-free activity: fold increase is 1.5 $\pm$ 0.2, $n = 3$. **, $P < 0.001$, Student's *t* test. *Bars*, SE.

Results

Increased MAPK Activity in T5-PRF Cells. Previously, we had shown that T5-PRF human breast cancer cells had an elevated level of basal ER activity (in the absence of estrogen), despite the fact that these cells contained 50% less ER[4] (9). We were interested in possible reasons for this apparently estrogen-independent ER activity. Several lines of evidence suggest that MAPK may be involved in ligand-independent activation of ER-α. Therefore, we chose to examine MAPK activity in T5-PRF and the parental T5 human breast cancer cell lines. Our initial experiments examining ER-α transcriptional activity were performed under basal (*i.e.*, estrogen-deplete) conditions with cells setup in PRF/DMEM containing twice charcoal-stripped FCS; therefore, we initially examined *in vitro* MAPK activity under these conditions (Fig. 1*A*). *In vitro* MAPK activity was significantly higher in T5-PRF human breast cancer cells. MAPK activity was 2.6 $\pm$ 0.3 (mean $\pm$ SE, $n = 3$) fold higher ($P < 0.05$) than the activity assayed in the parental T5 cells. To determine whether this increased activity was reflected in the total amount of MAPK protein and/or the active MAPK pool, Western blotting on cell extracts was performed (Fig. 2). Using an antibody that recognizes total MAPK protein, we found that both cell lines expressed large amounts of both the ERK1 and ERK2 isoforms of MAPK under these conditions (Fig. 2*B*). The active form of MAPK is dually phosphorylated on tyrosine and threonine residues (2), and using an antibody that specifically detects only this active form of the protein, we found that T5-PRF cells had higher levels of activated MAPK protein compared with parental cells (Fig. 2*A*). PD 98059 is a specific inhibitor of MAPK activity because of its specific inhibition of MAPK kinase (also referred to as MEK; Ref. 14). Treating both cell lines with 50 μM PD 98059 for 1 h resulted in a significant inhibition of dually phosphorylated MAPK (Fig. 2*A*), with little or no effect on total MAPK levels (Fig. 2*B*), supporting the conclusion that T5-PRF cells have elevated levels of activated MAPK. Under conditions in which we had serum starved the cells for 7 days, T5-PRF cells still maintained elevated levels of *in vitro* MAPK activity (1.5 $\pm$ 0.2, fold, mean $\pm$ SE, $n = 3$, $P < 0.001$) compared with parental T5 cells (Fig. 1*B*). Total MAPK protein levels were similar between the two lines (Fig. 3*B*), but activated MAPK protein levels were much higher in T5-PRF cells (Fig. 3*A*, compare *Lanes 1* and *3*). As expected, treating T5-PRF cells with 50 μM PD 98059 for 1 h under serum-free conditions decreased the level of active MAPK detected by Western blotting (Fig. 3*A*, compare *Lanes 3* and *4*), without effecting the total level of MAPK protein expression (Fig. 3*B*, compare *Lanes 3* and *4*). These data support the conclusion that T5-PRF cells contain increased levels of phosphorylated and active MAPK protein.

Antiestrogen Can Reduce Active MAPK Protein Levels. Previous research has suggested that ER-α can directly activate MAPK, and estrogen activation of MAPK can be blocked by antiestrogen in MCF-7 human breast cancer cells (15). T5-PRF cells contain elevated basal ER-α activity that can be inhibited by 85% after treating cells for 24 h with 1 μM ICI 164,384.[4] To examine what effect ICI 164,384 might have on MAPK protein levels under these conditions, T5-PRF cells were treated with 1 μM ICI 164,384 for 24 h, and levels of active and total MAPK protein were examined. ICI 164,384 was able to reduce the amount of active dually phosphorylated MAPK in estrogen-depleted conditions by 30% ($n = 4$), without any apparent effect on

[4] A. S. Coutts, E. Leygue, and L. Murphy. Variant estrogen receptor-α messenger RNA expression in hormone-independent human breast cancer cells, submitted for publication.

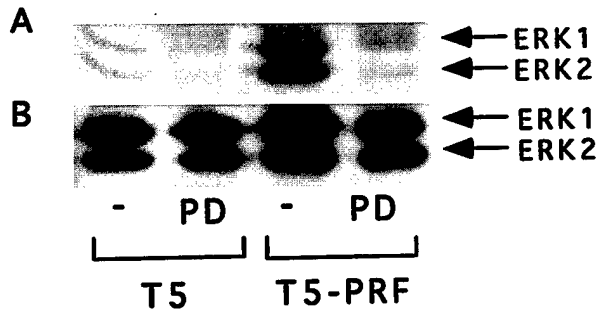


Fig. 2. Western blot analysis of MAPK protein levels under basal conditions. In *A*, 30 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-phospho-MAPK antibody (NEB). In *B*, 5 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-ERK1 antibody (Santa Cruz Biotechnology). *PD*, MEK inhibitor PD 98059, 50 μM for 1 h.

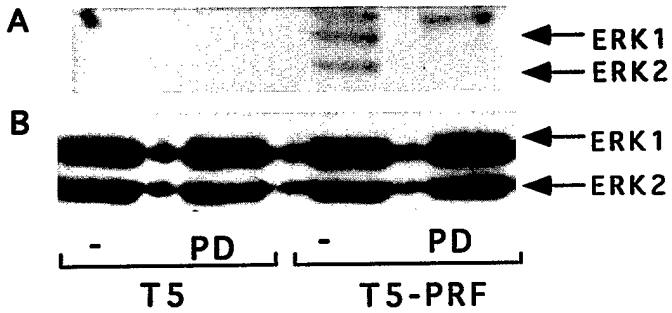


Fig. 3. Western blot analysis of MAPK protein levels under serum-free conditions. In *A*, 30 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-phospho-MAPK antibody (NEB). In *B*, 5 μg of cell extract were run on a 7.5% SDS-PAGE. Immune detection was using an anti-ERK1 antibody (Santa Cruz Biotechnology). *PD*, MEK inhibitor PD 98059, 50 μM for 1 h.

total MAPK levels (data not shown), suggesting a possible link between the elevated MAPK activity and increased ligand-independent ER-α activity in these cells.

MAPK Is Involved in ER-α Transcriptional Activity. To further examine the role elevated MAPK activity might play in terms of the elevated basal (*i.e.*, estrogen-independent) ER-α transcriptional activity in T5-PRF cells, transient transfections were carried out. Previously, we had determined that 50 μM PD 98059 for 1 h was sufficient to significantly reduce the levels of dually phosphorylated MAPK under basal conditions (Fig. 2*A*). Because our transient transfection experiments required 24-h treatments, we determined conditions under which PD 98059 was able to maintain low levels of dually phosphorylated MAPK over this time period (data not shown). It was determined that adequate inhibition could be achieved by treating cells initially with 50 μM PD 98059, followed 2 h later by an additional 50 μM treatment, with the cells being harvested 18 h later. This resulted in an ~85% inhibition of dually phosphorylated MAPK protein levels (data not shown). When T5-PRF cells were transfected with an estrogen-responsive CAT reporter gene and treated with PD 98059, there was a significant reduction of 44.2% $\pm$ 8.1 (mean $\pm$ SE, $P < 0.05$, $n = 6$) in the basal ER-α transcriptional activity seen (Fig. 4). Interestingly, there was also a significant and equivalent reduction in the estrogen-induced transcriptional activity of 46.3% $\pm$ 12.4 (mean $\pm$ SE, $P < 0.05$, $n = 6$) by PD 98059, suggesting a role for MAPK activation in estrogen-induced transcriptional activation also.

Discussion

We have found elevated levels of activated MAPK in a human breast cancer cell line, T5-PRF, that is nonresponsive to estrogen in terms of growth and has elevated levels of estrogen-independent ER transcriptional activity. The ER-α, like other members of the steroid hormone receptor superfamily, is a phosphoprotein. The function of phosphorylation is not clear, but it has been suggested to play a role in many aspects of receptor activity, including DNA binding and transcriptional activation. In MCF-7 human breast cancer cells, the ER-α is phosphorylated on serine-118, serine-154, and serine-167 in response to estradiol binding (6, 16, 17). In COS-1 cells, additional hormone-induced phosphorylation sites, serine-104, serine-106, and serine-118, on the ER-α have been identified (8). It has also been demonstrated that serine-118 and tyrosine-537 on ER-α are phosphorylated independently of estradiol binding in MCF-7 cells (5, 16) and that src family tyrosine kinases are capable of estradiol-independent phosphorylation of the ER-α *in vitro* (5). The activation of the MAPK pathway through an estrogen-independent mechanism (*i.e.*, EGF) can result in transcriptional activation of the ER-α, and phosphorylation of the ER-α on serine-118 is required for this activity (4, 7, 17).

Estrogens are known mitogens for breast cancer cells, but how estrogen promotes cell proliferation is unknown. The MAPK signal transduction pathway plays an essential role in cell cycle progression and can be activated by many growth factor/mitogenic pathways including estrogen. In several cell types, including MCF-7 human breast cancer cells, estradiol has been shown to rapidly increase MAPK activity (15). That this activation requires ER-α was demonstrated in cells via transient transfection experiments showing an absolute requirement for ER-α for activation, and the addition of antiestrogen blocked estrogen-induced MAPK activation (15). A recent report was able to show growth factor, but not estrogen-induced, activation of MAPK in MCF-7 cells (17). The discrepancy between this report and a previous report demonstrating estrogen activation of MAPK in MCF-7 cells (15) is unclear, but may be due to differences in the experimental conditions under which activation was assayed between the two papers.

Peptide growth factor signaling pathways can cross-talk with the ER-α. Indeed, it has been demonstrated that growth factors can result in ligand-independent activation of ER-α (7). Several studies have demonstrated that overexpression of a growth factor, or its receptor, which can activate the MAPK cascade, or a component of the MAPK pathway (*e.g.*, Raf) can result in estrogen-independent growth in cells in culture and in some cases tumorigenesis *in vivo* in the absence of estrogen. For example, overexpression of a constitutively active Raf

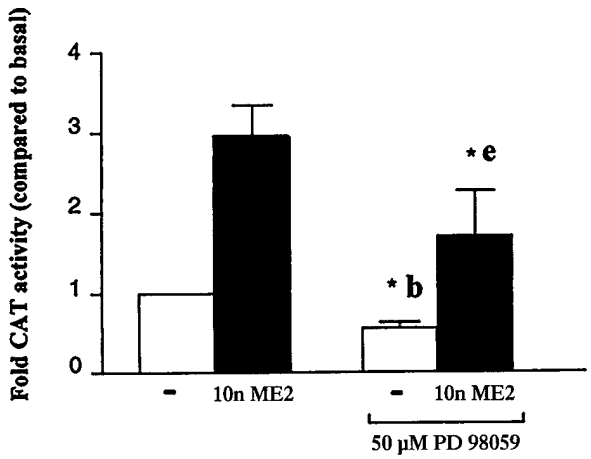


Fig. 4. ER-α transcriptional activity in the presence of PD 98059. Transient transfections were performed as described in "Materials and Methods." Results are expressed as fold CAT activity compared with untreated T5-PRF cells. Histograms represent means (*bars*, SE), $n = 6$. **b*, compared with basal without PD 98059; **e*, compared with estrogen treatment without PD 98059, $P < 0.05$, Student's t test.

kinase in MCF-7 cells allows for growth in the absence of estrogen (3). Expression of constitutively activated MEK in NIH 3T3 cells resulted in the ability of the cells to proliferate in conditions of low serum and also resulted in morphological transformation (18). These data demonstrate the importance of the MAPK pathway in cell growth regulation and likely tumorigenesis.

What role the elevated MAPK activity might play in the estrogen-independent growth phenotype of T5-PRF cells is not clear. Our previous studies have shown that these cells no longer respond to estrogen by increasing their proliferation rate. It is likely that elevated MAPK activity could confer an additional growth advantage to these cells, thereby allowing maximal proliferation in the absence of estrogen. Our transfection experiments using PD 98059 suggest that the MAPK activity may play a role in the elevated basal ER activity we see in these cells but may not account for all of the increased basal activity. At a concentration of 50 μM, PD 98059 can almost completely abolish the level of active dually phosphorylated MAPK protein (85% decrease), whereas a similar concentration resulted in slightly less than 50% reduction in basal ER-α transcriptional activity. This suggests that other mechanisms, along with elevated MAPK, contribute to the ligand-independent ER-α activity. Our previous study has identified a variant ER-α in T5-PRF cells that may contribute to the increased basal ER-α activity.[4] Studies have also shown that cells grown long-term in the absence of estrogen can develop supersensitivity to residual estrogens in the growth medium (19). It is also likely that the residual dually phosphorylated MAPK protein remaining, even after treating cells with PD 98059 (~15%), is sufficient to contribute to the basal ER-α activity.

We were also able to see an inhibition of estrogen-dependent ER-α transcriptional activity after treating cells with PD 98059. This was of a similar magnitude as the effect on basal transcription (~50%), supporting the hypothesis that MAPK plays an important role in both ligand-dependent and -independent ER-α transcriptional activity.

Our experiments with the antiestrogen ICI 164,384 also suggest that the link between MAPK activity and ER-α transcriptional activity is likely not a straightforward one. As mentioned, T5-PRF cells treated with 1 μM ICI 164,384 under basal conditions show an ~85% reduction in the ligand-independent ER-α transcriptional activity,[4] whereas the same dose and time of antiestrogen treatment results in an ~30% inhibition of dually phosphorylated MAPK protein.

Elevated levels of MAPK activity and expression have been associated with the malignant phenotype and have been shown in breast tumors compared with normal tissue and benign breast conditions (2). Breast tumors also have been shown to contain elevated tyrosine kinase activities compared with benign breast tumors and normal breast tissues (20). These data suggest that an increase or deregulation of growth controlling signals, such as those contributed by MAPK, may be involved in the etiology and pathogenesis of breast cancer. During the course of breast cancer progression, tumors become hormone-independent and refractory to endocrine therapies directed at blocking the activity of ER-α. The development of estrogen-independent growth is believed to be an initial step in the progression to a hormone-independent phenotype, and estrogen-independent growth is a characteristic of a more aggressive breast cancer cell phenotype. Our data support the hypothesis that elevated levels of MAPK could confer a growth advantage to breast cancer cells, perhaps leading to estrogen-independent growth.

Acknowledgments

We thank C. Richard (Department of Biochemistry and Molecular Biology, University of Manitoba) for help with the *in vitro* MAPK assays.

References

1. Seger, R., and Krebs, E. G. The MAPK signaling cascade. FASEB J., *9:* 726–735, 1995.
2. Sivaraman, V., Wang, H., Nuovo, G., and Malbon, C. Hyperexpression of mitogen-activated protein kinase in human breast cancer. J. Clin. Invest., *99:* 1478–1483, 1997.
3. El-Ashry, D., Miller, D., Kharbanda, S., Lippman, M., and Kern, F. Constitutive raf-1 kinase activity in breast cancer cells induces both estrogen-independent growth and apoptosis. Oncogene, *15:* 423–435, 1997.
4. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science (Washington DC), *270:* 1491–1494, 1995.
5. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. Phosphorylation of the human estrogen receptor on tyrosine 537 *in vivo* and by src family tyrosine kinases *in vitro*. Mol. Endocrinol., *9:* 24–33, 1995.
6. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. Mol. Endocrinol., *8:* 1208–1214, 1994.
7. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J., *15:* 2174–2183, 1996.
8. Le Goff, P., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J. Biol. Chem., *269:* 4458–4466, 1994.
9. Coutts, A. S., Davie, J. R., Dotzlaw, H., and Murphy, L. C. Estrogen regulation of nuclear matrix-intermediate filament proteins in human breast cancer cells. J. Cell. Biochem., *63:* 174–184, 1996.
10. Ahn, N. G., Weiel, J. E., Chan, C. P., and Krebs, E. G. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. J. Biol. Chem., *265:* 11487–11494, 1990.
11. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
12. Seiler-Tuyns, A., Walker, P., Martinex, E., Merillat, A. M., Givel, F., and Wahli, W. Identification of estrogen-responsive DNA sequences by transient expression experiments in a human breast cancer cell line. Nucleic Acids Res., *14:* 8755–8770, 1986.
13. Alkhalaf, M., Murphy, L. J., and Murphy, L. C. Enhanced c-jun activity alters responsiveness to medroxyprogesterone acetate in Ishikawa human endometrial carcinoma cells. Mol. Endocrinol., *7:* 1634–1641, 1993.
14. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. PD 98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J. Biol. Chem., *270:* 27489–27494, 1995.
15. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J., *15:* 1292–1300, 1996.
16. Arnold, S. F., Obourn, J. D., Yudt, M. R., Carter, T. H., and Notides, A. C. *In vivo* and *in vitro* phosphorylation of the human estrogen receptor. J. Steroid Biochem. Mol. Biol., *52:* 159–171, 1995.
17. Joel, P. B., Traish, A. M., and Lannigan, D. A. Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/44 mitogen-activated protein kinase. J. Biol. Chem., *273:* 13317–13323, 1998.
18. Greulich, H., and Erikson, R. L. An analysis of Mek1 signaling in cell proliferation and transformation. J. Biol. Chem., *273:* 13280–13288, 1998.
19. Masamura, S., Santner, S. J., Heitjan, D. F., and Santen, R. J. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J. Clin. Endocrinol. Metab., *80:* 2918–2925, 1995.
20. Hennipman, A., van Oirschot, B. A., Smits, J., Rijksen, G., and Stall, G. E. J. Tyrosine kinase activity in breast cancer, benign breast disease, and normal breast tissue. Cancer Res., *49:* 516–521, 1989.

APPENDIX 8

Influence of Estrogen Receptor mRNA Variants on the Determination of ER status in Human Breast Cancer

Aihua Huang, Etienne Leygue, Helmut Dotzlaw, Leigh C. Murphy, Peter H. Watson.

Affiliations of authors:

A. Huang, and P.H. Watson, (Department of Pathology), E. Leygue, H. Dotzlaw, and L.C. Murphy (Department of Biochemistry and Molecular Biology), University of Manitoba, Winnipeg, Manitoba, R3EOW3.

Grant support: This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). EL is a USAMRMC postdoctoral fellow. LCM is a Medical Research Council of Canada (MRC) Scientist. PHW is an MRC Clinician-Scientist.

Correspondence to: Dr. Peter Watson, Department of Pathology, Faculty of Medicine, D212-770 Bannatyne Ave, University of Manitoba, Winnipeg, MB, R3E 0W3, Canada.

Telephone: (204)-789-3435, Fax: (204)-789-3931, e-mail: pwatson@cc.umanitoba.ca

Keywords: Estrogen variant mRNAs, Estrogen receptor status, immunohistochemistry, breast cancer

Abstract

Background: **Estrogen receptor alpha (ER) status in breast cancer is an important predictive factor for clinical response to endocrine therapy. We have recently shown that discrepancies in ER status determined by immunohistochemical assay (ER-IHA) can occur between different ER antibodies and that this may be attributable to the expression of truncated ER variant proteins. In this study we have examined the direct effect of ER variant expression on ER-IHA and whether discrepancies in ER-IHA status are reproducible with different antibodies.**

Methods: **ER negative Cos-1 cells were transiently transfected with expression vectors containing wild type ER (wt-ER) and/or the truncated ER-Clone-4 variant. Expression of ER-like proteins was determined by Western blot and ER-IHA using ER specific monoclonal antibodies against different epitopes of the ER protein and quantitated by both densitometry and H-score analysis. Statistical comparisons were assessed by the student t-test. Paraffin sections from 10 tumors were also studied by ER-IHA.**

Results: **ER-IHA with N- and C-terminal ER antibodies on Cos-1 cells expressing wt-ER alone demonstrated no difference in signals by Western blot ($P>0.1$). However co-expression of wt-ER and the truncated ER-clone-4 variant, resulted in discordant IHA results with relatively higher ER-IHA scores from N-terminal antibodies ($P<0.03$). Furthermore, re-examination of breast tumors previously studied by ER-IHA showed persistent differences in 8/10 cases with a different pair of ER antibodies.**

Conclusions: **We conclude that truncated ER variant proteins can interfere with IHA determination of ER status and that this may account for some of the inconsistencies between ER status and response to endocrine therapy.**

Introduction

The measurement of estrogen receptor alpha (ER) status in breast tumors is widely used as a clinical index of potential therapeutic response to endocrine therapy. However, while up to 2/3 of breast tumors are ER positive, only 2/3 of this subset of patients will respond well to endocrine therapy (1). Several factors have been considered in the past to account for this discrepancy, including tissue related factors such as sampling, cellularity and heterogeneity and biological factors such as functional status of the ER protein detected and the integrity of the downstream components of the ER signalling pathway.

More recently other potentially important biological factors that have emerged are the complexity of ER alpha gene expression as well as the recent discovery of the closely related ER beta gene that is also expressed in breast cancer tissues (2). ER alpha gene expression is now known to be often associated with a range of ER variant mRNAs in breast tumors (3). These ER mRNA variants include exon-deleted, exon-duplicated or truncated ER mRNA transcripts that may encode a variety of incomplete ER-like proteins (3,4). Individual ER variant proteins have been demonstrated (5,6) but in most instances may well be expressed at low levels in most tumors compared to wt-ER (4). Nevertheless ER variant expression may be important to consider with the adoption of the IHA as an alternative to the classical ligand binding dextran coated charcoal (DCC) assay to determine ER status (7,8,9). The IHA affords the opportunity to determine ER status in paraffin tumor sections and so allows the parallel assessment of tissue factors. However this has also meant that ER is now defined on the basis of structural epitopes as opposed to functional ligand binding. Expression of most ER variants would not be detected by the DCC assay because in many cases the predicted variant proteins have loss or disruption of the C-terminus and ligand binding domain (E/F region) of the protein (10). However, the total accumulation of multiple ER-like proteins might well be

expected to interfere with IHA determination of ER status, depending on the target specificity of the antibody employed. Thus, although in practice a good overall correlation between IHA and DCC exists, discrepant results occur in a proportion of tumors (8,9,11,12,13). These discrepancies are not only between IHA and DCC assays (8,9), and also between IHA performed with different antibodies on the same tumors (11,12,13). While tissue related factors can be invoked to account for some of the former discrepancies, differences between comparable IHAs scored on the same areas within serial sections (11) are more difficult to explain.

In order to understand such discrepancies we have recently examined ER mRNA variant expression and shown that those variants that specifically encode putative truncated ER-like proteins, are preferentially expressed in these 'IHA-inconsistent' cases (14). This suggests that ER variant proteins endoed by ER variant mRNAs may contribute to discrepancies in ER status determined by IHA using different antibodies. In this study we have now compared the signal intensities of different ER targeted antibodies and used these to examine experimentally the direct effect of truncated ER variant expression on the determination of wild type ER (wt-ER) status by IHA.

Materials and Methods

ER expression vectors and transfection assays

Wt-ER (HEGO, kindly provided by Dr P. Chambon) was cloned into the vector pSG5 and expression was driven by an SV40 promotor. Truncated ER-clone-4 was cloned into the pcDNA3.1 vector (Invitrogen) and expression was driven by a CMV promotor. ER-negative Cos-1 cells were grown in DMEM supplemented with 5% (v/v) fetal bovine serum. The cells were transiently transfected with 5μg of either pHEGO or pER-clone-4 expression plasmid, or both in varying

proportions (as described in Fig 2). After 48 hours, transfected cells were harvested for immunoblotting or fixed for immunohistochemical assay. All transfections were done in 60-mm dishes (for Western blot samples) or in chamber slides (for immunohistochemistry samples) in parallel and using the Superfect transfection system (Qiagen, CA) as described by the manufacturer. Plasmid pCH110 (encoding β-galactosidase protein, Pharmacia) was co-transfected and galactosidase activity was determined by standard methods to control for and confirm transfection efficiency (25-35% cells positive).

Western Blot Analysis

Whole cell extracts were prepared from cells transfected with wt-ER, ER-clone-4 or both plasmids. Cells were washed with chilled phosphate-buffered saline (PBS), scraped, collected in PBS, centrifuged at 1000g for 5 minutes at 4^0C and cell pellets were resuspended in 200μl of 50 mM Tris-HCl, 20 mM EDTA, 5% Sodium dodecyl sulphate(SDS), 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM β-glycerophosphate and 1mM aprotinin. Protein concentration was determined by the Bio-Rad (Hercules, CA) protein assay kit as described by the manufacturer. Twenty-five micrograms of protein were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Western blot analysis to detect ER present in different transfected cells was carried out with ER-specific mouse monoclonal antibodies 1D5 (DAKO, Canada) or AER314 raised against N-terminal epitopes and AER311 or AER320 (Neomarkers, CA), raised against C-terminal epitopes (at 1/1000 dilution for all antibodies) of the wild type protein (Fig 1D). The second antibody used was a horse-radish peroxidase (HRP)-conjugated goat anti-mouse antibody (Hyclone Laboratories, Logan, UT, USA).Visualization was accomplished using the Supersignal detection system (Pierce, USA.) according to the manufacturer's instructions. Densitometry on Western blot signals was performed using a video-computer image analysis system (M4, Imaging

Research, St Catherines, Ontario). All Western blot experiments were performed at least in triplicate on independent cell transfections. Statisitcal comparisons between antibody signals were assessed by the student t-test.

Immunohistochemistry

In parallel experiments, Cos-1 cells were grown in chamber slides (Nalge Nunc, Intl., Il, USA). Transfections were performed as above. Cells were fixed in 2% paraformaldehyde-PBS for 30 minutes and washed twice with PBS for 2 minutes. Then IHA was performed as described previously (11). Briefly, the slides were incubated in 10% goat serum in PBS for 20 minutes to block non-specific binding. Primary antibody 1D5 or AER311 (at 1/50 dilution for both) was added and incubation carried out overnight at 4°C, followed by two washes in PBS for 5 minutes. The second antibody (biotinylated goat anti-mouse IgG, Vector Labs, CA) was used at 1:200 dilution in PBS for 45 minutes at room temperature. After a rinse in PBS, the slides were incubated in AB Complex (Elite kit, Vector Labs) at 1:100 dilution for 45 minutes. The label was developed using diaminobenzidine/hydrogen peroxide and slides were then lightly counterstained with methyl green, dehydrated, cleared and mounted.

Human breast tumor specimens (10 cases) were obtained from the NCIC-Manitoba Breast Tumor Bank. Formalin fixed and paraffin embedded tissue blocks were sectioned to provide serial sections from tumors and examined by IHA using the same protocol except that a different pair of ER antibodies (AER314 and AER320, Neomarkers, CA) were used.

Semi-quantitative H-scoring for all IHA experiments was done as previously described (11). Brown immunoreactivity of cell nuclei was taken as positive and the proportion of negative cells (P_0) and those staining at low (P_1), moderate (P_2), or high (P_3) levels of intensity scored. The score for each section (H-score = $[(0xP_0) + (1xP_1) + (2xP_2) + (3xP_3)] \times 100$) was calculated from the

mean of x 5 representative high power fields (Leica DMRB, x40 objective). For transfection experiments where non-specific background was uniformly higher than in tumor sections, only P_2 and P_3 values were entered into the final H-score. Initial IHA experiments were also analyzed with the video-densitometry system as above, to compare with and validate the H-scoring quantification. All IHA slides were coded and assessment was done without knowledge of the antibody, transfection conditions, or tumor identity. As described previously (11), tumors which exhibited an H-score difference of >50 between IHA assays performed with different ER antibodies on serial sections were classified as 'IHA-inconsistent'.

Results.

Comparison of ER antibodies.

A panel of ER antibodies directed to different epitopes was tested by IHA and Western blot assay in parallel, applied to an ER negative Cos-1 cell line transfected with wt-ER. All antibodies detected a 65kDa protein on Western blot (Fig.1A) and comparison using a video-densitometry system showed that there was no significant difference between the signals obtained with 1D5 and AER311 antibodies (optical density units $mean^{SD}$ for 1D5 = $0.79^{0.22}$ vs $mean^{SD}$ AER311 = $0.78^{0.19}$; n = 4, P>0.1). IHA was performed in parallel on transfected cells using these antibodies at the same relative concentrations. These were also the concentrations that we had previously used to study breast tumors. Initially IHA was assessed by both video-densitometry and semi-quantitative H-score (applied independently and without knowledge of the antibody) and it was found that these provided comparable results (n = 10, r = 0.96, p = 0.004, data not shown). Subsequently all IHA signals were quantitated by H-score. Positive immunoreactivity in approximately 30% of nuclei was seen with IHA-1D5 and IHA-AER311 with no significant difference in ER levels (H-score 1D5 $mean^{SD} = 63^{8}$; AER311 $mean^{SD} = 60^{17}$, n = 7, P>0.1, Fig.2D).

Effect of modulation of ER variant expression on IHA.

The ER-clone-4 variant was used for these experiments as this variant is predicted to encode a C-terminally truncated ER-like protein and has previously been shown to be frequently expressed in breast tumors (14,15,16). Following transfection of ER-clone-4 alone, a single 24 kDa protein was detected by Western blot analysis (Fig.1B) and positive nuclear staining was only seen by IHA using N-terminal antibodies (1D5 and AER314). However, C-terminal antibodies (AER311 and AER320) failed to detect it on Western blot (Fig 1B&C) or IHA (Fig.2D). IHA was then performed using the same protocol as used in our previous study of breast tumors applied to cells following co-transfection with ER-clone-4 variant and wt-ER to obtain different proportions of ER variant expression relative to wt-ER. We observed that while both antibodies recognized wt-ER on Western blot (Fig.1C) and gave similar H-score values with wt-ER alone, a significant and increasing discordance in IHA H-score occurred between 1D5 and AER311 as the relative proportion of ER-clone-4 variant increased ($P<0.03$, t-test, Fig 2D).

ER-IHA on breast tumors.

We then investigated the reproducibility of our original observation in breast tumors. A different pair of antibodies was selected for repeat IHA assay (AER314 and AER320 targeting N- and C-terminal ER epitopes respectively) as these were found to give similar results in Cos-1 cells following wt-ER transfection and Western blot analysis (AER314 $mean^{SD}=0.67^{0.28}$;vs AER320 $mean^{SD}=0.65^{0.22}$; n=4, $P>0.1$) and IHA (data not shown). The original tissue blocks were available on 10 cases that were all ER/PR positive by DCC assay. All 5 tumors that had previously been classified as 'IHA-inconsistent' by our previous definition (H-score difference >50) showed lower H-scores by IHA-AER320 as compared to IHA with the matching AER314

antibody. In 3/5 this was sufficient to remain classified as 'IHA-inconsistent' (Table 1). In contrast, amongst an equal number of cases previously classified as 'IHA-consistent', 3/5 showed equivalent or higher H-scores with AER320. In 1/5 of the latter cases the IHA was inconsistent.

Discussion.

Multiple ER alpha mRNA variants are expressed in normal breast tissue and in breast tumors (3). However, in considering the role of ER variants in breast cancer, it has been argued that expression of ER mRNA variants may not be important on the basis that expression may not change during tumorigenesis and that the evidence to date for expression of specific variant proteins that might play a role in hormonal progression is limited (17,18). Nevertheless, studies founded on histologically characterised tissue sections have clearly shown that certain mRNA variants are differentially expressed between normal and neoplastic tissues and also between tumors (19,20). Differential expression has also been found in association with contrasting responses to estrogen and resistance to tamoxifen in cell lines (21,22,23,24) and parameters of hormone response and prognosis in-vivo (16,25). Overexpression of an ER mRNA variant deleted in exon 5 (D5-ER) has also been shown to occur in certain tamoxifen resistant tumors (26) and has been used successfully to predict reponse to hormonal therapy of hepatocellular carcinoma (27). It is also conceivable that ER variants might exert indirect functional effects through competition with wt-ER fof ER binding proteins (28) or with proteins involved in interactions with antiestrogens (29) .

Although expression of specific variant proteins has not yet been proven in breast tumors, partly because it has been difficult to develop antibodies that will distinguish variants, expression of ER-like variant proteins expressed recombinantly can be demonstrated in-vitro and in breast cell lines (21,22). In some cases these variant proteins have been shown to possess either hormone independent and constitutive activity or to exert a dominant negative influence on estrogen regulated target genes

in ex-vitro models. At the same time our own data suggest that previous attempts to demonstrate these proteins in-vivo may have been hampered by the fact that, although total expression of ER variants of all forms may be significant, many individual variants, such as D5-ER may be expressed at only low levels in breast tissues (4). Furthermore, our recent observation that inconsistent immunostaining with ER antibodies correlates with the total overal expression of mRNA variants encoding out-of-frame proteins (i.e. predicted to encode C-terminally truncated ER-like proteins) also supports the view that ER-like variant proteins are present in-vivo (11,14). In the latter study we examined ER expression in breast tumors by DCC and IHA using both 'N-terminal' (1D5) and 'C-terminal' (AER-311) targeting ER alpha antibodies. The IHA provided similar results to the DCC assay in terms of ER status. However the ER-IHA results from almost 25% of tumors were discordant (H-score difference of > 50) between these different antibodies, even when scored on the same areas within serial sections (11). Further analysis of these 'IHA-inconsistent' cases by RT-PCR assays (4,20) showed that those ER mRNA variants that encode putative truncated ER-like proteins, were preferentially expressed in 'IHA-inconsistent' cases (14).

However, although the antibodies we used provided similar signal intensities by IHA on strongly ER positive tumors, and in many tumors the IHA signals were concordant (11), it remained possible that the discordant IHA signals might be explained by different antibody affinities. Furthermore, the principle that ER variants may interfere with IHA results had not previously been tested experimentally. Our results here show that the 1D5 and AER-311 antibodies can provide similar signal intensities of wt-ER by both Western blot and IHA when used at the same relative concentrations as in our previous study (11). But the relative IHA signal intensity obtained with these antibodies changes and becomes inconsistent with increasing expression of a truncated ER variant protein alongside the wt-ER. IHA consistency or inconsistency is also reproducible when tumor blocks that have previously been studied are re-

examined using a different pair of matched ER monoclonal antibodies targeting similar N and C terminal epitopes. In one case the paired IHA results were quite different between these studies. However the precise epitopes recognized by all the ER antibodies used is unknown. Also the presence of additional truncated variants in this particular case, similar to ER-clone-4, that would remain undetected by the RT-PCR assays we have previously used, cannot be ruled out (15,16).

In summary, we have shown that ER status determined by IHA can be directly influenced by expression of a C-terminally truncated ER variant and that inconsistent IHA results in tumors are reproducible. We conclude that ER variant proteins can interfere with IHA assessment of ER status and that this may underlie some of the inconsistencies in determination of ER status in breast tumors. The relationship between ER variant expression, 'IHA-inconsistent' status and clinical response to endocrine therapy remains to be determined.

Figure Legends

Figure 1. Western blot analysis of estrogen receptor expression detected with different antibodies in Cos-1 cells following transient transfection with ER and/or ER-clone-4 variant. Twenty five μg of whole cell extract protein prepared from transfected Cos-1 cells were loaded in each lane and separated by 0.1%SDS-10%PAGE. (A) Cos-1 cells transfected with wt-ER. (B) Cos-1 cells transfected with ER-clone-4 (C) Cos-1 cells transfected with both wt-ER and ER-clone-4. Monoclonal antibodies used to detect ER proteins were 1D5 or AER314, and AER311 or AER320, which target epitopes within the N-terminal and C-terminal of the ER protein, respectively (D).

Figure 2 Immunohistochemical detection of estrogen receptor–like protein in transfected Cos-1 cells. IHA-1D5 assay (A, B) and IHA-AER311 assay (C) were conducted on cells transfected with either ER-wt alone (A), or co-transfected with ER-wt and ER-clone-4 (ratio 1%:99%, B&C). The graph (D) summarizes the H-score values obtained by either IHA-1D5 or IHA-AER311 applied to Cos-1 cells transfected with ER-wt and ER-clone-4 in different proportions. Each bar represents the mean and standard deviation relative to 1D5 H-score applied to ER-wt transfected cells derived from 7 (ER-wt alone) or 3 (all other) independent transfection experiments.

REFERENCES:

1. McGuire WL. Hormone receptors: their role in predicting response to endocrine therapy. Semin Oncol 1978;5:428-33.
2. Dotzlaw H, Leygue E, Watson PH, Murphy LC. Expression of estrogen receptor-beta in human breast tumors. J Clin Endocrinol Metab 1997;82:2371-4.
3. Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH. Oestrogen receptor variants and mutations in human breast cancer. Ann Med 1997;29:221-34.
4. Leygue E, Huang A, Murphy L, Watson P. Prevalence of estrogen receptor variant mRNAs in human breast cancer. Cancer Res 1996;56:4324-7.
5. Park W, Choi JJ, Hwang ES, Lee JH. Identification of a variant estrogen receptor lacking exon 4 and Its coexpression with wild-type estrogen receptor in ovarian carcinomas. Clin Cancer Res 1996;2:2029-35.
6. Desai AJ, Luqmani YA, Walters JE, Coope RC, Dagg B, Gomm JJ, et al. Presence of exon 5-deleted oestrogen receptor in human breast cancer: functional analysis and clinical significance. Brit J Cancer 1997;75:1173-1184.
7. Taylor CR. Paraffin section immunocytochemistry for estrogen receptor. Editorial Cancer 1996;77:2419-22.
8. Allred DC, Bustamante MA, Daniel CO, Gaskill HV, Cruz AJ. Immunocytochemical analysis of estrogen receptor in human breast carcinomas. Evaluation of 130 cases and review of the literature regarding concordance with biochemical assay and clinical relevance. Arch Surg 1990;125:107-113.
9. Alberts SR, Ingle JN, Roche PR, Cha SS, Wold LE, Farr GH, et al. Comparison of estrogen receptor determinations by a biochemical ligand assay and immunohistochemical staining with

monoclonal antibody ER1D5 in females with lymph node positive breast carcinoma entered on two prospective clinical trials. Cancer 1996;78:764-72.

10. Dowsett M, Daffada A, Chan CMW, Johnston SRD. Oestrogen Receptor mutants and variants in breast cancer. Euro J Cancer 1997;33:1177-1183.
11. Huang A, Pettigrew N, Watson P. Immunohistochemical assay for estrogen receptors in paraffin wax sections of breast carcinoma using a new monoclonal antibody. J Pathol 1996;180:223-7.
12. Pertschuk LP, Feldman JG, Kim YD, Braithwaite L, Schneider F, Braverman AS, et al. Estrogen receptor immunocytochemistry in paraffin embedded tissues with ER1D5 predicts breast cancer endocrine response more accurately than H222Spγ in frozen sections or cytosol-based ligand-binding assays. Cancer 1996;77:2514-9.
13. Nedergaard L, Christensen L, Rasmussen BB, Jacobsen GK. Comparison of two monoclonal antibodies for the detection of estrogen receptors in primary breast carcinomas Pathol Res Pract 1996;192:983-8.
14. Huang A, Leygue E, Snell L, Murphy L, Watson P. Expression of estrogen receptor variant mRNAs and determination of estrogen receptor status in human breast cancer. Am J Pathol 1997;150:1827-33.
15. Dotzlaw H, Alkhalaf M, Murphy L. Characterization of estrogen receptor variant mRNAs from breast cancers. Mol Endocrinol 1992;6:773-85.
16. Murphy LC, Hilsenbeck SG, Dotzlaw H, Fuqua SAW. Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. Clin Cancer Res 1995;1:155-9.
17. Pfeffer U, Fecarotta E, Arena G, Forlani A, Vidali G. Alternate splicing of the estrogen receptor priamry transcript normally occurs in estrogen receptor positive tissues and cell lines. J Steroid

Biochem 1996;56:99-105.

18. Gotteland M, Desauty G, Delarue JC, Liu L, May E. Human estrogen receptor messenger RNA variants in both normal and tumor tissues. Mol Cell Endocrinol 1995;112:1-13.

19. Leygue E, Watson P, Murphy L. Estrogen receptor variants in normal human mammary tissue. J Natl Cancer Inst 1996;88:284-90.

20. Leygue E, Murphy L, Watson P. Triple primer polymerase chain reaction: a new way to quantify truncated mRNA expression. Am J Pathol 1996;148:1097-103.

21. Castles CG, Fuqua SAW, Klotz DM, Hill SM. Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. Cancer Res 1993;53:5934-9.

22. Pink JJ, Wu SQ, Wolf DM, Bilimoria MM, Jordan VC. A novel 80 kDa human estrogen receptor containing a duplication of exons 6 and 7. Nucleic Acids Res 1996;24:962-9.

23. Madsen MW, Reiter BE, Lykkesfeldt AE. Differential expression of estrogen receptor mRNA splice variants in the tamoxifen resistant human breast cancer cell line, MCF-7/TAMR-1 compared to the parental MCF-7 cell line. Mol Cell Endocrinol 1995;109:197-207.

24. Castles CG, Klotz DM, Fuqua SAW, Hill SM. Coexpression of wild type and variant estrogen receptor mRNAs in a panel of human breast cell lines. Br J Cancer 1995;71:974-80.

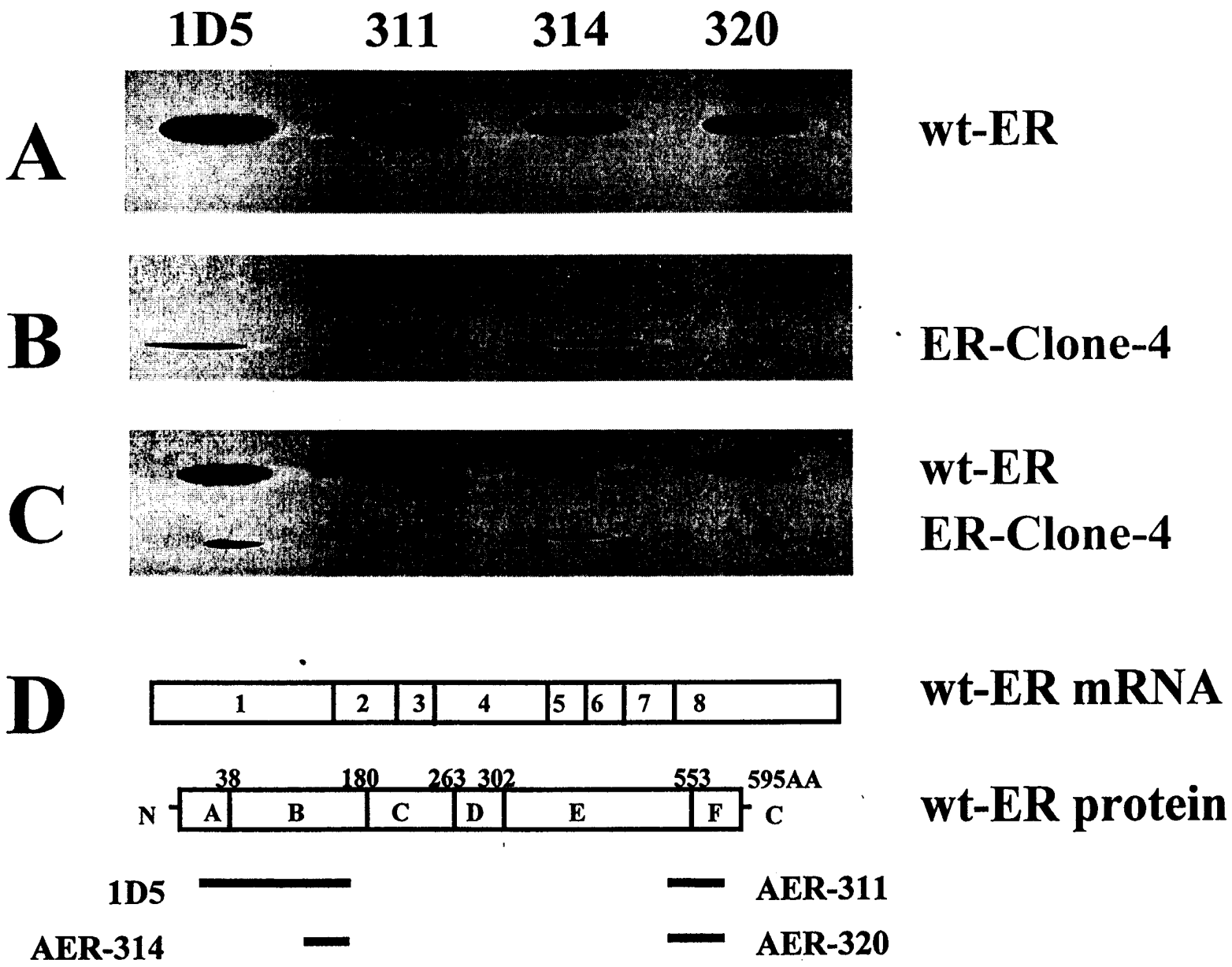
25. Fuqua SAW, Chamness GC, McGuire WL. Estrogen receptor mutations in breast cancer. J Cell Biochem 1993;51:135-9.

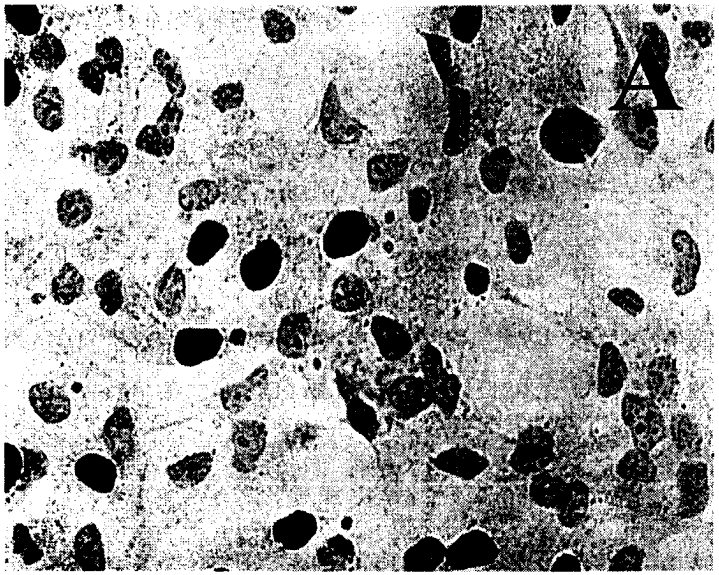
26. Daffada AA, Johnston SR, Smith IE, Detre S, King N, Dowsett M. Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. Cancer Res 1995;55:288-93.

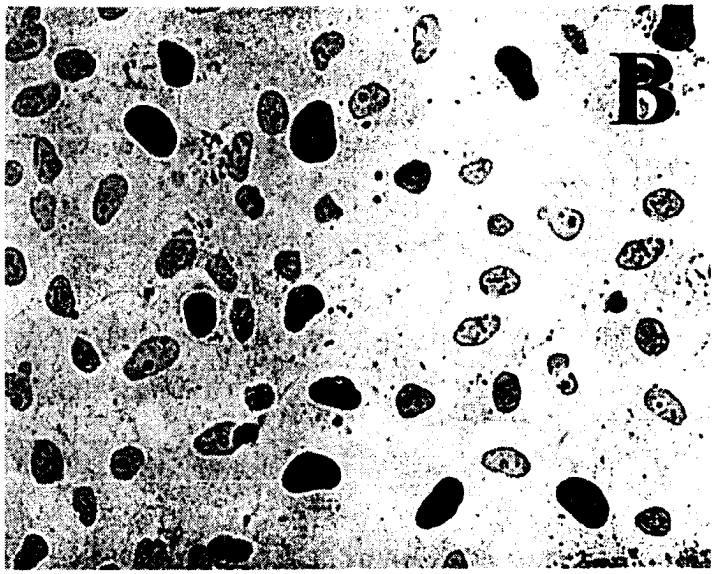
27. Villa E, Dugani A, Fantoni E, Camellini L, Buttafoco P, Grottola A, et al. Type of estrogen receptor determines response to antiestrogen therapy. Cancer Res 1996;56:3883-5.

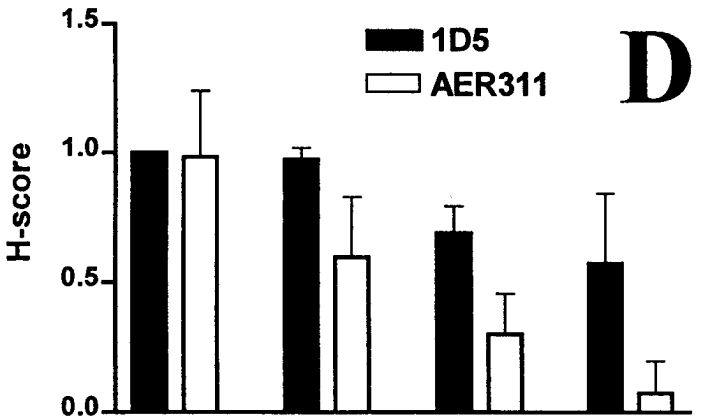
28. Halachmi S, Marden E, Martin G, Mackay H, Abbondanza C, Brown M. Estrogen receptor associated proteins: possible mediators of hormone induced transcrition. Science 1994;264:1455-8.

29. Yang NN, Venugopalan M, Hardikar S, Glasebrook A. Identification of an estrogen response element activated by metabolites of estradiol and raloxifene. Science 1996;273:1222-5.


1D5
311
314
320
A
wt-ER
B
ER-Clone-4
C
wt-ER
ER-Clone-4
D
1
2
3
4
5
6
7
8
wt-ER mRNA
38
180
263
302
553
595AA
N
A
B
C
D
E
F
C
wt-ER protein
1D5
AER-311
AER-314
AER-320







ER-wt	100%	10%	1%	0
ER-clone4	0	90%	99%	100%

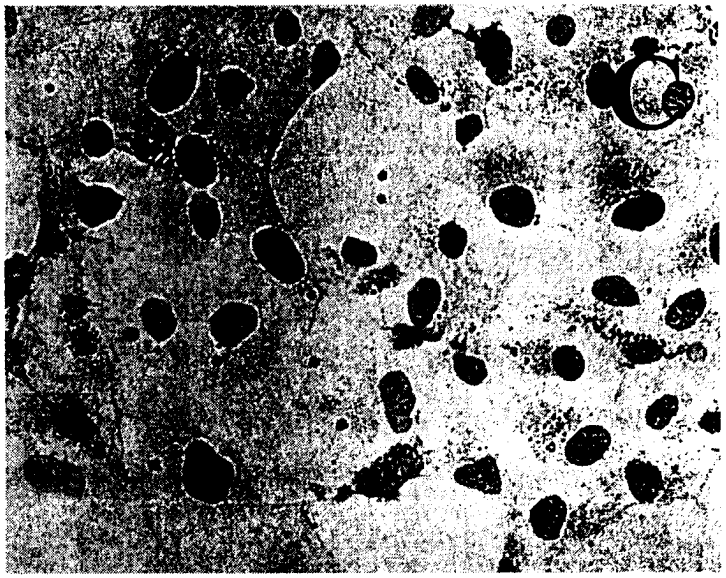


Table 1 H-Score determination by immunohistochemical assay with different ER antibodies

Tumor #	*ER*	*PR*	*1D5*	*AER311*	*1D5↔311*	*AER314*	*AER320*	*314↔320*
1	69	18	200	100	**100**	150	80	**70**
2	7	26	134	14	**120**	180	90	**90**
3	33	21	182	88	**94**	110	60	**50**
4	140	101	160	66	**94**	125	100	25
5	19	10	190	20	**170**	90	80	10
6	39	44	132	128	4	105	140	35
7	79	22	124	138	14	150	170	20
8	85	125	120	150	30	150	150	0
9	27	10	228	200	28	165	135	25
10	24	59	128	104	24	150	50	**100**

ER / PR: estrogen / progesterone receptor level determined by DCC assay (fmol/mg protein)

1D5, AER311, AER314, AER320: estrogen receptor level (H score values) determined with the corresponding antibody.

1D5↔311, 314↔320: Difference in H-score values with each antibody pair. Inconsistent H-Score between antibodies (difference >=50) shown in bold, consistent H-Score between antibodies (<50) shown in regular typeface.

APPENDIX 9

Pergamon

PII: S0960-0760(98)00012-0

J. Steroid Biochem. Molec. Biol. Vol. 65, No. 1–6, pp. 175–180, 1998

Printed in Great Britain
0960-0760/98 $19.00 + 0.00

The Pathophysiological Role of Estrogen Receptor Variants in Human Breast Cancer

Leigh C. Murphy*, Helmut Dotzlaw, Etienne Leygue, Amanda Coutts and Peter Watson

Department of Biochemistry and Molecular Biology, University of Manitoba, 770 Bannatyne Ave, Winnipeg, Canada R3E 0W3

The accumulated evidence supports the expression of estrogen receptor variants at both the mRNA and protein levels. The relative level of expression of some estrogen receptor variant mRNAs and possibly progesterone receptor variant mRNAs is altered during breast tumorigenesis and breast cancer progression. The altered expression of estrogen receptor variants may effect estrogen signal transduction as well as the interpretation of assays where the estimation of estrogen receptor levels is used as a guide to treatment strategies and prognosis. © 1998 Elsevier Science Ltd. All rights reserved.

J. Steroid Biochem. Molec. Biol., Vol. 65, No. 1–6, pp. 175–180, 1998

INTRODUCTION

The estrogen receptor is considered pivotal in the mechanism by which estrogen interacts with its target cells and mediates its specific effects. Classically, the estrogen receptor is considered to be a ligand activated transcription factor, which upon estrogen binding undergoes conformational changes which allow it to dimerize, tightly bind to estrogen responsive DNA sequences and alter transcription of target genes [1, 2]. However, the diverse effects of estrogen on target tissues [3–6] and the observation that many human breast cancers develop estrogen independence despite the continued expression of the estrogen receptor [7] suggest that the concept of estrogen action described above is unlikely to simply explain all aspects of estrogen action [2, 8]. Evidence has accumulated over the last decade supporting the existence of estrogen receptor variants [9, 10]. Therefore the possibility exists that estrogen receptor variants may have a pathophysiological role in estrogen action. For example, the pattern of estrogen receptor variant expression may influence which set of estrogen responsive genes are transcribed. The following discussion reviews the evidence available to support a pathophysiological role of estrogen receptor variants in human breast tissues.

STRUCTURE OF ESTROGEN RECEPTOR VARIANT MRNAS

Most data supporting the existence of estrogen receptor (ER) variants have been at the mRNA level. Two main structural patterns of estrogen receptor variant mRNAs have been consistently identified: the truncated ER mRNAs [11] and the deleted ER mRNAs [12]. Using a long range reverse transcription-polymerase chain reaction analysis (RT-PCR) [13] which detects all deleted ER variant mRNAs at a frequency relative to their initial mRNA representation in the unamplified sample [14], the most frequently detected and likely the most abundant deleted ER variant mRNAs in human breast tumors appear to be the exon 7 deleted ER mRNA [13, 15] and the exon 4 deleted ER mRNA [13, 16]. However, in some breast tumors other deletion variants such as an exon 3 + 4 deleted ER mRNA [13] and an exon 4 + 7 deleted ER mRNA [13] have been frequently detected. An exon 5 deleted ER variant mRNA is rarely detected using the long range RT-PCR approach, suggesting that its abundance is low compared to several other ER deleted mRNAs. However, when specific PCR primers are used to measure only the exon 5 deleted ER mRNA relative to the wild-type ER mRNA, the levels of the exon 5 variant mRNA are found to vary

Proceedings of the 13th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology "*Recent Advances in Steroid Biochemistry & Molecular Biology*" Monaco 25–28 May 1997.

*Correspondence to L. C. Murphy. Tel.: +1-204-7893233; fax: +001-204-789-3900; e-mail: lcmurph@cc.umanitoba.ca.

amongst breast tumor samples [17] as well as between normal breast tissues and breast tumors [18]. Also truncated ER variant mRNAs have been frequently detected at relatively high abundance in several human breast tumors [19, 20]. In fact the truncated ER variant mRNAs are the only ER variant transcripts that have been detected by Northern blotting analysis and the entire cDNAs cloned and sequenced [11, 19]. A commonly expressed truncated ER mRNA is the clone 4 truncated ER mRNA. The predicted proteins of these relatively most abundant ER variant transcripts are shown schematically in

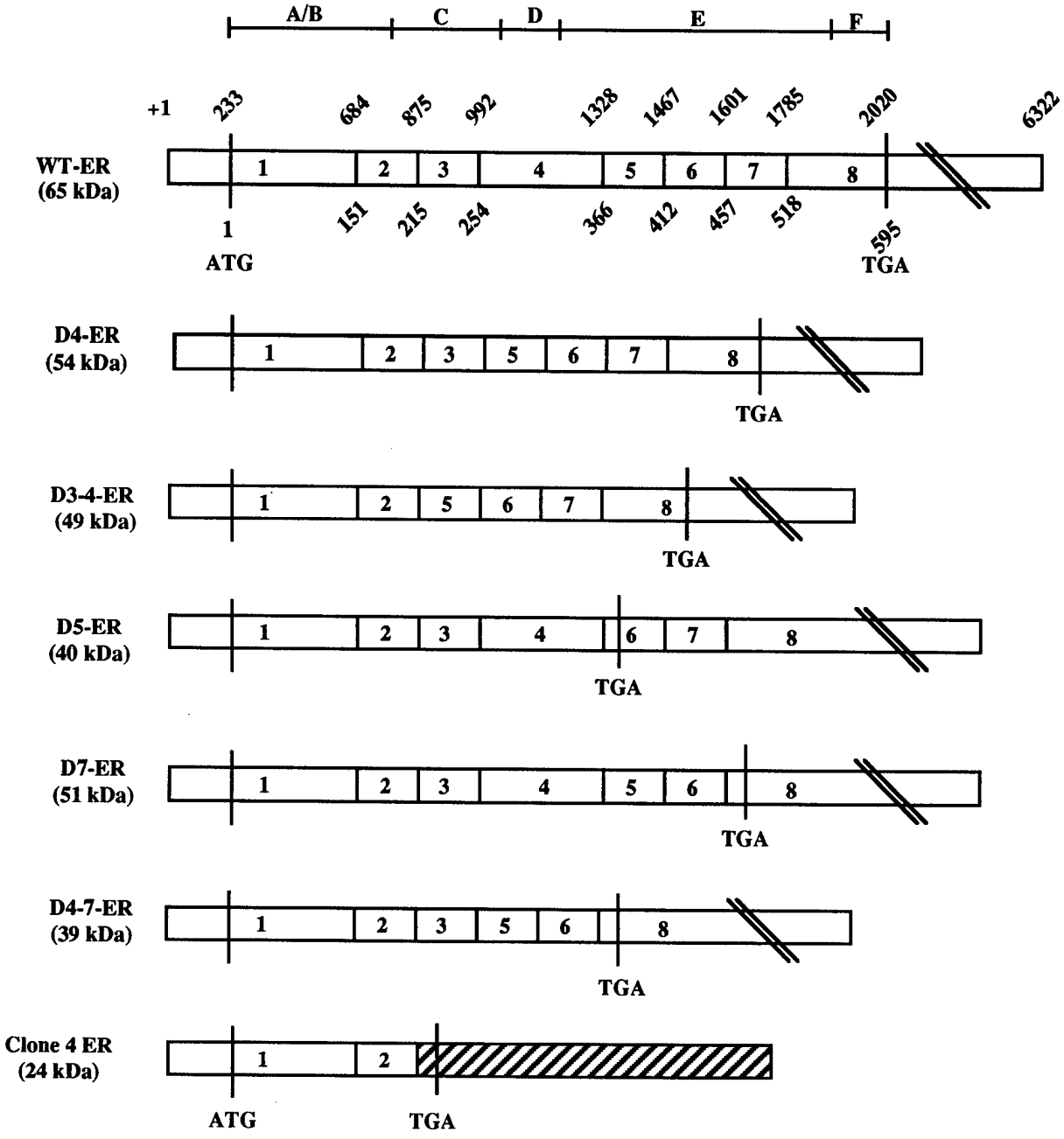


Fig. 1. Schematic diagram of the wild-type human estrogen receptor (ER) cDNA, which contains 8 different exons coding for a protein divided into structural and functional domains (A–F). Region A/B of the receptor is implicated in trans-activating function (AF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (AF-2). The numbering on the top of the cDNA refers to the nucleotide position as defined in reference [39]. Below the wild-type ER cDNA are the various putative exon deleted and truncated ER cDNAs. ATG shows the translation initiation codons, TGA shows the inframe translation termination codons and the numbering below the cDNA refers to the amino acid positions as defined in Ref. [39]. D = deletion, and the estimated molecular mass (kDa = kiloDaltons) of each open reading frame is shown in brackets. Molecular masses were estimated using MacVector version 4.1.4 software.

Fig. 1. All of these variant transcripts will encode ER proteins missing some structural/functional domains of the wild-type ER. In many cases i.e. the exon 7 deleted, the exon 4 + 7 deleted and the clone 4 truncated ER variant mRNAs will encode C-terminally truncated proteins in which the ligand binding domain, the ligand dependent transcriptional activity (AF-2) and the ligand dependent dimerization domain are significantly impaired. Although the exon 4 deleted and the exon 3 + 4 deleted transcripts are inframe, studies indicate that the proteins encoded by these transcripts cannot bind ligand and have little, if any, ligand dependent transcriptional activity [16, 21]. Furthermore, the proteins encoded by the clone 4 truncated, the exon 3 deleted, the exon 3 + 4 deleted and the exon 4 + 7 deleted variant mRNAs are all unlikely to bind significantly to classical estrogen responsive DNA sequences. A common feature of all the relatively abundantly expressed ER variant mRNAs described above, is they would encode proteins with intact A/B regions. This region has been described to contain a promoter and cell-type specific transcriptional activity [22, 23], although its ability to function in the complete absence of an intact DNA binding domain is unexplored. In summary, a large body of molecular data exists to support the potential expression of ER variant proteins.

SPECIFICITY OF ESTROGEN RECEPTOR VARIANT EXPRESSION

The available studies provide evidence for an extensive and complex pattern of alternative splicing associated with the estrogen receptor gene, which appears to be altered during breast tumorigenesis. It has been suggested that the complex pattern of exon deleted ER variant mRNA expression is specific for the estrogen receptor, since similar variants for the glucocorticoid receptor and the retinoic acid receptors alpha and gamma have not been found in breast tumor tissues [24]. We have also investigated the pattern of exon deleted variant mRNA expression in breast tumors using a long range RT-PCR approach [13] for the progesterone receptor (PR) [25],

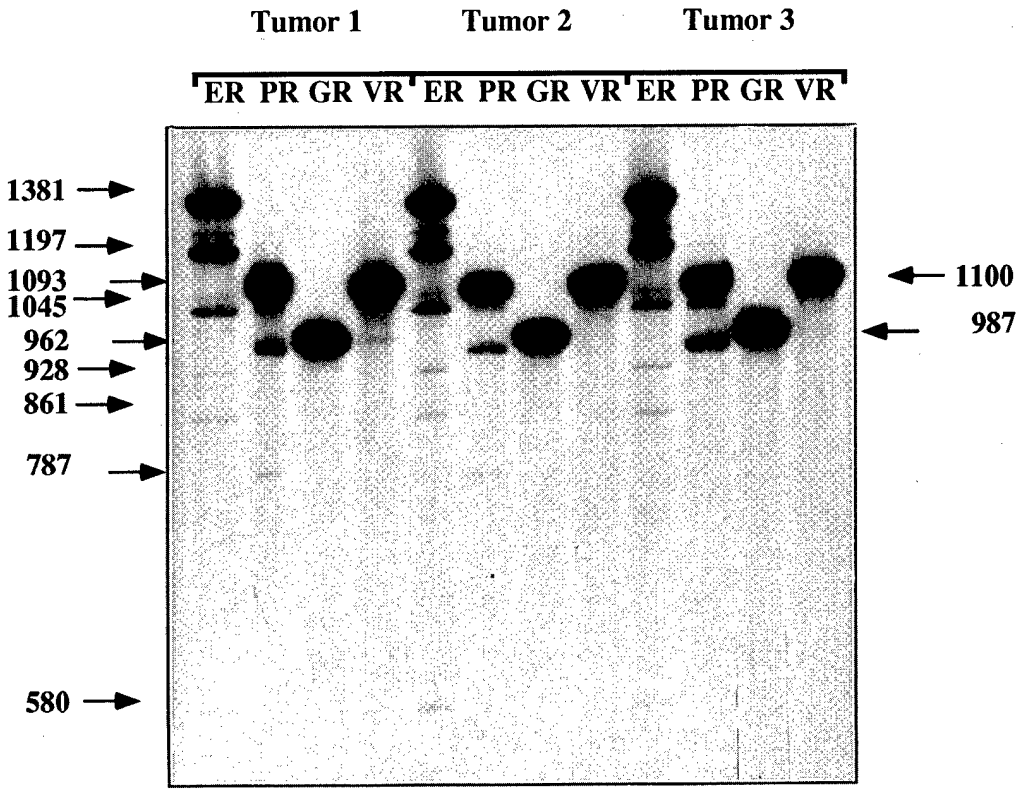


Fig. 2. Autoradiograph of long range RT-PCR analysis for ER, PR, GR and VR mRNA isolated from three human breast tumor biopsy samples. All tumors were ER+ and PR+ by ligand binding assay. The primers and RT-PCR conditions for ER and PR were as previously described [13, 25] except that the annealing time was 30 seconds only. The primers for the GR are as previously described [24] and located in exons 2 and 8, respectively. The RT-PCR conditions were those described above for ER and PR. The primers for the VR are: VR-U 5′-GAAGCGGAAGGCACTAT-3′, sense 155–171 as defined in [27]; VR-L 5′-GAGCACAAGGGGCGTTA-3′, antisense 1240–1256 as defined in Ref. [27]. D = deletion. The numbers beside the arrows represent the sizes (bp) of the PCR product: 1381 is WT-ER; 1197 is D7-ER; 1100 is WT-VR; 1093 is WT-PR; 1045 is D4-ER; 987 is WT-GR; 962 is D6-PR; 928 is D3 + 4-ER; 861 is D4 + 7 ER; 787 is D4-PR; 580 is D3 + 7-ER [13, 25].

the glucocorticoid receptor (GR) [26] and the vitamin D3 receptor (VR) [27]. Our data, shown in Fig. 2, demonstrate that little, if any, deleted variant mRNAs for the GR and VR were detected in the three breast tumors examined. However, in these same breast tumors, several exon deleted variant mRNA species for both the ER and PR were abundantly expressed (Fig. 2). Importantly, our published data have shown that the expression of the exon 5 deleted ER variant mRNA and the truncated clone 4 ER variant mRNA is elevated in breast tumors compared to normal breast tissues [18, 28] and our preliminary data suggest that the level of the exon 6 deleted PR variant mRNA was more highly expressed relative to the wild-type PR mRNA in breast tumors compared to normal breast tissues (Leygue *et al.* unpublished data). These data suggest that the mechanisms generating alternatively spliced forms of both ER and PR are unlikely to be due to a generalized deregulation and/or alteration of splicing processes within breast tumors. They also suggest that the mechanism(s) is specific for the sex steroid hormone receptor genes and the alterations seen in breast tumors may have a role in altered actions of estrogens and progestins in human breast tumorigenesis.

IDENTIFICATION OF ESTROGEN RECEPTOR VARIANT PROTEINS

Recent data published from several independent groups strongly support the detection of estrogen receptor like proteins which could correspond to some of the previously identified estrogen receptor variant mRNAs in both cell lines and tissues in vivo.

An ER-like protein consistent with that predicted to be encoded by the exon 5 deleted ER transcript is expressed naturally in some BT 20 human breast cancer cell lines [29]. A monoclonal antibody specific to the predicted unique C-terminal amino acids of an exon 5 deleted ER protein was developed, and used to demonstrate immunohistochemically the presence of this protein in several human breast cancer samples [30]. An exon 4 deleted ER variant mRNA has been identified in both normal and neoplastic ovarian tissue [31]. Western blotting analyses revealed the presence of the expected 65 kDa wild-type ER protein as well as a 53 kDa protein which was recognized by ER antibodies to epitopes in the N-terminus and C-terminus of the wild-type protein, but not with an antibody recognizing an epitope encoded by exon 4 [31]. These data strongly support the hypothesis that the small sized ER-like protein in the ovarian tissue extracts was encoded by an exon 4 deleted ER variant mRNA.

Recently, we have analyzed several human breast tumors immunohistochemically for ER expression, comparing side-by-side an antibody recognizing an N-terminally localized epitope in the wild-type ER protein (1D5), and an antibody recognizing a C-terminally localized epitope in the wild-type ER protein (AER311). It was found that although in many tumors the immunohistochemical results using each antibody showed good concordance with each other, in some tumors the results were discordant, with the signal tending to be higher with the N-terminal antibody. Since many of the proteins predicted from variant ER mRNAs would be truncated at the C-terminus and not contain the epitope recognized by the C-terminal antibody, one interpretation of these data would be that truncated variant ER proteins are more highly expressed in the discordant group of tumors [32]. This hypothesis was tested by investigating the pattern and relative expression of variant ER mRNAs in the discordant and concordant groups of breast tumors. Several ER variant mRNAs (the clone 4 truncated ER mRNA, the exon 2, 3 + 7 deleted ER mRNA, the exon 2, 3 + 4 deleted ER mRNA and the variant deleted within exon 3 to within exon 7) which encode putative truncated ER-like proteins that would be recognized only by an N-terminally targeted antibody were preferentially and more highly expressed in the discordant breast tumor group [33]. While this indirect approach does not specifically identify ER variant proteins, the data suggest that the ER variant mRNAs encoding truncated ER proteins may contribute to discrepancies in ER measured by immunodetection assays using N- or C-terminal antibodies.

The accumulated data provide strong support for the ability of, at least some ER variant mRNAs to be stably translated into proteins detectable by conventional methodologies. Such proteins may have a functional role in altered estrogen signal transduction in human breast tumorigenesis.

FUNCTIONAL SIGNIFICANCE OF ESTROGEN RECEPTOR VARIANT EXPRESSION

The important question to be addressed now is the role of ER variants in those tissues in which they are expressed. One approach has been to recombinantly express individual ER variants, alone and together with the wild-type ER, and determine effects on transcriptional activity as measured by a classical estrogen response element (ERE)-reporter gene. This has demonstrated the exon 5 deleted ER to have constitutive activity [34] and wild-type ER inhibitory activity [30]. As well the exon 3 deleted ER [35], the exon 4 deleted ER [31] and the exon 7 deleted ER [15] have been identified in some systems to have wild-type ER inhibitory activity. Some ER variants such as the exon 3 + 4 deleted variant have been shown to enhance wild-type ER activity, at least at the basal level [36]. Other ER variants such as the truncated clone 4 ER have no detectable activity in similar assays [11].

Another approach has been to determine if the ER variants are differentially expressed in normal vs neoplastic breast tissues. The accumulated data support the increased expression of the exon 5 deleted [18, 31] and the clone 4 truncated ER mRNAs [28] in breast tumors with good prognositic features compared to normal breast tissues. Furthermore it has been suggested that decreased expression of an exon 3 deleted ER mRNA occurs in breast tumors compared to normal breast tissue [37]. Further changes seem to occur during the later stages of breast cancer progression since the relative level of expression of the clone 4 truncated ER mRNA was found significantly elevated in breast tumors with characteristics of poor prognosis and endocrine resistance vs those with characteristics of good prognosis and endocrine sensitivity [20]. Moreover, increased expression of an exon 3 + 4 deleted ER was found associated with the estrogen independent, ER positive phenotype in a breast cancer cell line model [36] and preliminary data support the functional involvement of the overexpressed ER variant in the estrogen independent phenotype (Coutts *et al.*, unpublished data).

Inconsistent results have been obtained with respect to stably overexpressing an individual ER variant in a hormone dependent breast cancer cell line and the resulting development of endocrine resistance [10, 38]. The data suggest that altered ER variant expression may contribute to altered estrogen receptor activity which together with other factors will contribute to breast cancer progression and the eventual development of hormone independence and resistance to endocrine therapy [7].

CONCLUSIONS

Estrogen receptor variants can be detected at both the mRNA and protein levels. The level of expression of some ER variants is altered during breast tumorigenesis and breast cancer progression. The functional involvement of ER variants and possibly PR variants in breast cancer progression and altered responses to these steroid hormones requires further detailed investigation. More immediately, the impact of expression of variant ER and PR on the determination of ER and PR immunohistochemically as markers of prognosis and treatment response in breast cancer requires assessment.

Acknowledgements—This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). L. C. M. is a Medical Research Council of Canada (MRC) Scientist, P. H. W. is a MRC Clinician–Scientist, E. L. is a recipient of a USAMRMC Postdoctoral Fellowship, A. C. is a recipient of a Manitoba Health Research Council (MHRC) Studentship.

REFERENCES

1. Tsai M. J. and O'Malley B. W., Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63** (1994) 451–486.
2. Katzenellenbogen B. S., Estrogen receptors: Bioactivities and interactions with cell signaling pathways. *Biol. Reprod.* **54** (1996) 287–293.
3. George, F. W. and Wilson, J. D. Sex determination and differentiation. In *The Physiology of Reproduction*, ed. E. Knobil and J. D. Neill. Raven Press, New York, 1988, pp. 3.
4. Spicer, D. and Malcolm, C. Epidemiology of breast cancer. In *Treatment of the postmenopausal woman: basic and clinical aspects*, ed. R. Lobo. Raven Press, New York, 1994, pp. 315–324.
5. Turner R., Riggs B. and Spelsberg T., Skeletal effects of estrogens. *Endocr. Rev.* **15** (1994) 275–300.
6. Barrett-Conner E. and Bush T., Estrogens and coronary heart disease in women. *J. Am. Med. Ass.* **265** (1991) 1861–1867.
7. Murphy L., Mechanisms associated with the progression of breast cancer from hormone dependence to independence. *Pezcoller Found. J.* **2** (1995) 6–16.
8. Katzenellenbogen B. S. and Korach K. S., A new actor in the estrogen receptor drama-enter ER-beta. *Endocrinology* **138** (1997) 861–862editorial.
9. Murphy, L., Leygue, E., Dotzlaw, H., Douglas, D., Coutts, A. and Watson, P. Oestrogen receptor variants and mutations in human breast cancer. *Ann. Med.*, **29** (1997) 221–234.
10. Fuqua S. A. and Wolf D. M., Molecular aspects of estrogen receptor variants in breast cancer. *Breast Cancer Res. Treat.* **35** (1995) 233–241.
11. Dotzlaw H., Alkhalaf M. and Murphy L. C., Characterization of estrogen receptor variant mRNAs from human breast cancers. *Mol. Endocrinol.* **6** (1992) 773–785.
12. Fuqua S. A., Allred D. C. and Auchus R. J., Expression of estrogen receptor variants. *J. Cell. Biochem. Suppl.* **17G** (1993) 194–197.
13. Leygue E., Huang A., Murphy L. and Watson P., Prevalence of estrogen receptor variant messenger RNAs in human breast cancer. *Cancer Res.* **56** (1996) 4324–4327.
14. Fasco M., Quantitation of estrogen receptor mRNA and its alternatively spliced mRNAs in breast tumor cells and tissues. *Anal. Biochem.* **245** (1997) 167–178.
15. Fuqua S. A., Fitzgerald S. D., Allred D. C., Elledge R. M., Nawaz Z., McDonnell D. P., O'Malley B. W., Greene G. L. and McGuire W. L., Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. *Cancer Res.* **52** (1992) 483–486.
16. Pfeffer U., Fecarotta E., Arena G., Forlani A. and Vidali G., Alternative splicing of the estrogen receptor primary transcript normally occurs in estrogen receptor positive tissues and cell lines. *J. Steroid Biochem. Mol. Biol.* **56** (1996) 99–105.
17. Daffada A. A., Johnston S. R., Smith I. E., Detre S., King N. and Dowsett M., Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. *Cancer Res.* **55** (1995) 288–293.
18. Leygue E. R., Watson P. H. and Murphy L. C., Estrogen receptor variants in normal human mammary tissue. *J. Natl. Cancer Inst.* **88** (1996) 284–290.
19. Murphy L. C. and Dotzlaw H., Variant estrogen receptor mRNA species detected in human breast cancer biopsy samples. *Mol. Endocrinol.* **3** (1989) 687–693.
20. Murphy L. C., Hilsenbeck S. G., Dotzlaw H. and Fuqua S. A. W., Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. *Clin. Cancer Res.* **1** (1995) 155–159.
21. Inoue S., Hoshino S., Miyoshi H., Akishita M., Hosoi T., Orimo H. and Ouchi Y., Identification of a novel isoform of estrogen receptor, a potential inhibitor of estrogen action, in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **219** (1996) 766–772.
22. Tzukerman M., Esty A., Santiso-Mere D., Danielian P., Parker M., Stein R., Pike J. and McDonnell D., Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol. Endocrinol.* **8** (1994) 21–30.

23. Berry M., Metzger D. and Chambon P., Role of the two activating domains of the oestrogen receptor in the cell type and promoter context dependent agonistic activity of the antioestrogen 4 hydroxytamoxifen. *EMBO J.* **9** (1990) 2811–2818.
24. Pfeffer U., Fecarotta E. and Vidali G., Coexpression of multiple estrogen receptor variant messenger RNAs in normal and neoplastic breast tissues and in MCF-7 cells. *Cancer Res.* **55** (1995) 2158–2165.
25. Leygue E., Dotzlaw H., Watson P. H. and Murphy L. C., Identification of novel exon-deleted progesterone receptor variant mRNAs in human breast tissue. *Biochem. Biophys. Res. Commun.* **228** (1996) 63–68.
26. Hollenberg S., Weinberger C., Ong E., Cerelli G., Oro A., Lebo R., Thompson E., Rosenfeld M. and Evans R., Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318** (1985) 635–641.
27. Baker A., McDonnell D., Hughes M., Crisp T., Mangelsdorf D., Haussler M., Pike J., Shine J. and O'Malley B., Cloning and expression of full length cDNA encoding human vitamin D receptor. *Proc. Natl. Acad. Sci. USA* **85** (1988) 3294–3298.
28. Leygue E., Murphy L., Kuttenn F. and Watson P., Triple primer polymerase chain reaction. A new way to quantify truncated mRNA expression. *Am. J. Pathol.* **148** (1996) 1097–1103.
29. Castles C. G., Fuqua S. A., Klotz D. M. and Hill S. M., Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. *Cancer Res.* **53** (1993) 5934–5939.
30. Desai A., Luqmani Y., Coope R., Dagg B., Gomm J., Pace P., Rees C., Thirunavukkarasu V., Shousha S., Groome N., Coombes R. and Ali S., Presence of exon 5 deleted oestrogen receptor in human breast cancer: Functional analysis and clinical significance. *Br. J. Cancer* **75** (1997) 1173–1184.
31. Park W., Choi J., Hwang E. and Lee J., Identification of a variant estrogen receptor lacking exon 4 and its coexpression with wild type estrogen receptor in ovarian carcinomas. *Clin. Cancer Res.* **2** (1996) 2029–2035.
32. Huang A., Pettigrew N. and Watson P., Immunohistochemical assay for oestrogen receptors in paraffin wax sections of breast carcinoma using a new monoclonal antibody. *J. Pathol.* **180** (1996) 223–227.
33. Huang, A., Leygue, E., Snell, L., Murphy, L., and Watson, P. Expression of estrogen receptor variants mRNAs and determination of estrogen status in human breast cancer. *American Journal of Pathology*, **150** (1997) 1827–1833.
34. Fuqua S. A., Fitzgerald S. D., Chamness G. C., Tandon A. K., McDonnell D. P., Nawaz Z., O'Malley B. W. and McGuire W. L., Variant human breast tumor estrogen receptor with constitutive transcriptional activity. *Cancer Res.* **51** (1991) 105–109.
35. Wang Y. and Miksicek R. J., Identification of a dominant negative form of the human estrogen receptor. *Mol. Endocrinol.* **5** (1991) 1707–1715.
36. Coutts, A., Leygue, E., and Murphy, L. Mechanisms of hormone independence in human breast cancer cells. Abstract 1988. In *88th Annual Meeting of the American Association for Cancer Research*. San Diego, CA, 1997, pp. 296.
37. Erenburg, I., Mira-y-Lopez, R., Ossowski, L., and Schachter, B. Effects of estrogen receptor splice isoform on mammary cell function. Abstract P2-794. In *The 10th International Congress of Endocrinology*. San Francisco, 1996.
38. Rea D. and Parker M. G., Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. *Cancer Res.* **56** (1996) 1556–1563.
39. Green S., Walter P., Kumar V., Krust A., Bornert J., Argos P. and Chambon P., Human estrogen receptor cDNA: sequence, expression, and homology to v-erb-A. *Nature* **320** (1986) 134–139.

APPENDIX 10

ALTERED EXPRESSION OF ESTROGEN RECEPTOR ALPHA VARIANT mRNAs BETWEEN ADJACENT NORMAL BREAST AND BREAST TUMOR TISSUES

Etienne Leygue, Helmut Dotzlaw, Peter H. Watson, Leigh C. Murphy

Affiliations of authors: Department of Biochemistry and Molecular Biology [EL, HD, LCM] and Department of Pathology [PHW], University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3EOW3.

Running title: ER variant mRNAs in breast tissue.

This work was supported by a grant from the U.S. Army Medical Research and Materiel Command (DAMD17-95-1-5015). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). EL is a recipient of a U.S. Army Medical Research and Materiel Command Postdoctoral Fellowship (DAMD17-96-1-6174), PHW is a Medical Research Council of Canada (MRC) Clinician-Scientist, LCM is an MRC Scientist.

Abstract

Several variant forms of the estrogen receptor alpha (ER-α) mRNA have been described in human breast tissues. Among these, ER-α mRNA variants truncated after sequences encoding exon 2 of the wild-type ER-α mRNA (ERC4 mRNA), or deleted in exon 3 (ERD3 mRNA) or exon 5 (ERD5 mRNA) sequences, were previously shown to be differentially expressed between independent normal and breast tumor tissues. Using semi-quantitative reverse transcription-polymerase chain reaction assays, we have investigated the expression of these variant mRNAs relative to wild-type ER-α mRNA in 18 samples of normal breast tissues and their adjacent matched breast tumor tissues. A general trend towards a higher ERC4 mRNA and a lower ERD3 mRNA relative expression in the tumor compartment was observed. These differences reached statistical significance when considering only the ER-positive/progesterone receptor positive ($p=0.019$) and the ER-positive ($p=0.023$) subsets, as measured by the ligand binding assay, respectively. A significantly ($p=0.035$) higher relative expression of ERD5 mRNA was observed in tumor components overall. These data confirm previous observations and demonstrate that changes in the expression of ER-α variant mRNAs relative to wild-type occur between adjacent normal and neoplastic breast tissues.

Key words

Estrogen receptor, breast cancer, tumor progression, variant mRNAs.

Introduction

Estrogen receptors alpha (ER-α) and beta (ER-β) are thought to mediate the action of estradiol in target tissues (1, 2). These two receptors, which belong to the steroid/retinoic acid/thyroid receptor superfamily (3), contain several structural and functional domains (4) encoded by two mRNAs containing 8 exons (5, 6). Upon ligand binding, ER-α and ER-β proteins recognize specific estrogen responsive elements (EREs) located in DNA in the proximity of target genes, and through interactions with several co-activators modulate the transcription of these genes (7, 8). Several ER-α and ER-β variant mRNAs have been identified in both normal and neoplastic human tissues (for a review see 9, 10-13). Most of these variants contain a deletion of one or more exons of the wild-type ER mRNA. The putative proteins encoded by these variant mRNAs would therefore be missing some functional domains of the wild-type receptors and might interfere with wild-type ER signaling pathways. Indeed,

in vitro functional studies have shown that some recombinant ER-α variant proteins can affect estrogen regulated gene transcription. For example, ERD3, the variant protein encoded by exon 3 deleted ER-α mRNA, that is missing the second zinc finger of the DNA binding domain, has been shown to have a dominant negative activity on wild-type ER-α receptor action (14). A similar dominant negative activity has been observed for ERD5 variant protein, encoded by an ER-α variant mRNA deleted in exon 5 sequences, that is missing a part of the hormone binding domain of the wild-type molecule (15). Interestingly, a constitutive hormone independent activity (16) and a wild-type enhancing activity (17) have also been attributed to ERD5 variant protein in different systems. The relevance of the levels achieved in these transfection experiments to *in vivo* expression remains unclear. One should also note that these functional activities are likely to be cell type and promoter specific (9). However, the discovery that these ER-α variants are expressed in both normal and neoplastic human breast tissues raised the question of their possible role in breast tumorigenesis (see ref 9 and references herein). We have previously reported an increased relative expression of ERD5 mRNA and of ERC4 mRNA, another ER-α variant mRNA truncated of all sequences following the exon 2 of the wild-type ER-α, in breast tumor samples versus independent normal breast tissues (18-20). In contrast, Erenburg et al. reported recently a decreased relative expression of ERD3 mRNA in tumor tissues and cancer cell lines versus independent normal reduction mammoplasty samples (21). These data, which suggested that alteration in ERD5, ERD3 and clone 4 mRNA expression may occur during breast tumorigenesis, were obtained in tissues from different individuals and possible inter-individual differences cannot be excluded. In order to clarify this issue, we investigated the expression of these three variant mRNAs in normal breast tissues and their matched adjacent primary breast tumor tissues.

Materials and methods

Human breast tissues and reverse transcription

Human breast specimens (18 cases) were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The processing of specimens collected in the Manitoba Breast Tumor Bank has already been described (22). Briefly, each specimen had been rapidly frozen as soon as possible after surgical removal. A portion of the frozen tissue block was processed to create a paraffin-embedded tissue block matched and orientated relative to the remaining frozen block. These paraffin blocks provide high quality histologic sections, which are used for

pathologic interpretation and assessment and are mirror images of the frozen sections used for RNA extractions. For each case, tumor and adjacent normal tissues from the same individual were histologically characterized by observation of paraffin sections. The presence of normal ducts and lobules as well as the absence of any proliferative lesion were confirmed in all 18 normal specimens. Within the eighteen adjacent primary invasive ductal breast carcinomas, seven were ER negative (< 3 fmol/mg protein, mean = 1.44 fmol/mg protein) and eleven were ER positive (> 3 fmol/mg protein, ranging from 3.5 to 159 fmol/mg protein, mean = 38.8 fmol/mg protein), as determined by the ligand binding assay. Within the ER negative tumor subgroup, 6 tumors were progesterone receptor (PR) negative (<10 fmol/mg protein, mean = 6.41 fmol/mg protein) and 1 was PR positive (>10 fmol/mg protein), as measured by the ligand binding assay. In the ER positive subgroup, 2 tumors were PR negative and 9 were PR positive (PR levels ranging from 11.6 to 134 fmol/mg protein, mean = 52.6 fmol/mg protein). Total RNA was extracted from frozen tissue sections and reverse transcribed in a final volume of 25 µl as previously described (18). The quality of cDNAs obtained was assessed by amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, as described previously (18).

Polymerase chain reaction (PCR)

ERC4 primer set was used to co-amplify wild-type ER-α (WT-ER) and ERC4 cDNAs. This set consisted of ERU primer (5'-TGTGCAATGACTATGCTTCA-3'; sense; located in WT-ER exon 2; position 792-811, as numbered in reference 1), ERL primer (5'-GCTCTTCCTCCTGTTTTTAT-3'; antisense; located in WT-ER exon 3; position 940-921, as numbered in reference 1), and C4L primer (5'-TTTCAGTCTTCAGATACCCCAG-3'; antisense; located in C4ER sequence; position 1336-1315, as numbered in reference 19).

ERD3 primer set, used to co-amplify WT-ER and ERD3 cDNAs, consisted of D3U primer (5'-TGTGCAATGACTATGCTTCA-3'; sense; located in WT-ER exon 2; position 792-811, as numbered in reference 1) and D3L primer (5'-TGTTCTTCTTAGAGCGTTTGA-3'; antisense; located in WT-ER exon 4; position 1145-1125, as numbered in reference 1).

ERD5 primer set, used to co-amplify WT-ER and ERD5 cDNAs, consisted of D5U primer (5'-CAGGGGTGAAGTGGGGTCTGCTG-3'; sense; located in WT-ER exon 4; position 1060-1082, as numbered in reference 1) and D5L primer (5'-ATGCGGAACCGAGATGATGTAGC-3'; antisense; located in WT-ER exon 6; position 1542-1520, as numbered in reference 1).

PCR amplifications were performed and PCR products analyzed as previously described (18, 20, 23). Briefly, 0.2 µl of reverse transcription mixture was amplified in a final volume of 15 µl, in the presence of 1.5 µCi of [α-^{32}P] dCTP (3000Ci/mmol), 4 ng/µl of each primer of the primer set considered (ERC4, ERD3 or ERD5 primer set) and 0.3 unit of Taq DNA polymerase. Each PCR consisted of 30 cycles: 1 min at 94°C, 30 sec at 60°C and 1 min at 72°C for ERC4 primer set; and 30 sec 94°C, 30 sec at 60°C and 30 sec at 72°C for ERD3 and ERD5 primer sets. PCR products were then separated on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and autoradiographed. For each PCR, two PCR products were obtained, which were identified by subcloning and sequencing, performed as previously described (18, 23). PCR products migrating with the apparent size of 149 bp, 354 bp and 483 bp, using ERC4, ERD3 and ERD5 primer set, respectively, were shown to correspond to WT-ER cDNA. PCR products migrating with the apparent size of 536 bp, 237 bp and 344 bp, using ERC4, ERD3 and ERD5 primer set, were shown to correspond to ERC4, ERD3 and ERD5 cDNAs, respectively.

For each experiment, bands corresponding to the variant mRNA (i.e ERC4, ERD3 or ERD5) and to WT-ER, were excised from the gel and counted in a scintillation counter.

Quantitation and statistical analysis

For each set of primers (i.e ERC4, ERD3 and ERD5 primer set) and for each sample, 4 independent PCR assays were performed. The ratios between ERC4, ERD3 or ERD5 signals and corresponding WT-ER signal were calculated. For each experiment, in order to correct for overall inter-assay variations (due to different batches of radiolabelled [α-^{32}P] dCTP or of Taq DNA polymerase), the ratio observed in the same particular tumor (case number 12) was arbitrarily given the value of one and all other ratios expressed relatively, in arbitrary units (see Fig. 2). Under our experimental conditions, some samples did not have measurable levels (i.e signal lower than twice the background value) of D3ER or D5ER variant mRNAs (see figure 1 and 2) in any of the 4 repetitions performed. Only cases having detectable levels in at least 3 of the replicates in both their normal and tumor compartments were included in the statistical analysis. The significance of the differences in the relative levels of expression of ERC4, ERD3 and ERD5 mRNAs between matched normal and tumor components was determined using the Wilcoxon signed rank test.

Results

In order to investigate the expression of ERC4, ERD3 and ERD5 mRNA expression relative to WT-ER mRNA within matched normal and breast tumor tissues, eighteen cases were selected in the Manitoba Breast Tumor Bank, which had well separated and histopathologically characterized normal and adjacent neoplastic components. Total RNA was extracted from frozen tissue sections and reverse-transcribed as described in the "Materials and Methods" section. In order to assess the quality of the resulting cDNAs, PCR was performed using primers recognizing the ubiquitously expressed gene, GAPDH. Similar GAPDH signals were obtained in all samples, indicating a similar quality of all cDNA samples (data not shown).

Relative expression of ERC4 mRNA in matched normal and breast tumor tissues

A recently described triple-primer PCR assay (TP-PCR assay) was used to compare the relative expression of ERC4 mRNA between adjacent normal and tumor components (20, 23). In this assay, three primers are used simultaneously during the PCR: the upper primer is able to recognize both WT-ER and ERC4 cDNA sequences whereas the two lower primers are specific for each cDNA. Competitive amplification of two PCR products occurs, giving a final PCR-product ratio related to the initial input of target cDNAs. This approach has been validated previously both by competitive amplification of spiked cDNA preparations (20) and by comparison to RNase protection assays (23). As shown Fig. 1A, two main PCR products were obtained, that migrated at the apparent size of 149 bp and 536 bp. These products have been shown to correspond to WT-ER and ERC4 mRNAs, respectively (23). One should note the presence, in samples where WT-ER and ERC4 signals are high (see Fig. 1, lane 5), of several additional bands, one of which has been previously identified as corresponding to exon 2-duplicated ER-α variant cDNA (23). The presence of these minor PCR products did not interfer with the quantitative aspect of the TP-PCR assay (23). For each case, the mean of the ratios obtained in at least three independent PCR experiments, expressed in arbitrary units, is shown for both normal and tumor compartments (Fig. 2A). A general trend toward a higher clone 4 mRNA relative expression in the tumor compartment was observed (12 out of 18 cases, $p=0.47$, Wilcoxon signed rank test). When considering only the ER positive/PR positive subset ($n=9$), as measured by the ligand binding assay, a statistically higher ERC4 mRNA relative expression was found in the neoplastic components, as compared to matched adjacent normal tissues ($p=0.019$, Wilcoxon signed rank test).

Relative expression of ERD3 mRNA in matched normal and breast tumor tissues

A PCR assay, performed using primers annealing to sequences in exon 2 and 4, was used to investigate ERD3 mRNA expression relative to WT-ER in these 18 matched cases. We and others have previously shown that the co-amplification of WT-ER and an exon-deleted ER-α variant cDNA, resulted in the amplification of two PCR products, the relative signal intensity of which provided a previously validated measurement of exon-deleted ER-α variant expression (18, 24). Two PCR products, that migrated with an apparent size of 354 bp and 237 bp, were obtained (Fig. 1B). These fragments were shown by subcloning and sequencing to correspond to WT-ER and ERD3 mRNAs (data not shown). The relative ERD3 signal was measurable in the normal and the tumor compartments of 13 cases (see Fig. 2B). Out of these 13 cases, ERD3 mRNA expression was higher in the normal compartment of 10 cases. This difference, however, did not reach statistical significance ($p=0.057$, Wilcoxon signed rank test). A significantly higher expression of ERD3 mRNA in the normal compared to the adjacent neoplastic components was found when only the ER positive subset was considered ($n=8$, $p=0.023$, Wilcoxon signed rank test).

Relative expression of ERD5 mRNA in matched normal and breast tumor tissues

Using primers annealing to sequences in exon 4 and 6 of WT-ER, we also investigated the relative expression of ERD5 mRNA in these 18 matched cases. Two PCR products were obtained, that migrated at an apparent size of 483 bp and 344 bp and that have previously been shown to correspond to WT-ER and ERD5 cDNAs, respectively (Fig. 1C). As shown in Fig. 2C, a statistically significant higher relative expression of ERD5 mRNA was observed in tumor components when this expression was measurable in both normal and adjacent tumor tissues ($n=15$, $p=0.035$, Wilcoxon signed rank test).

Discussion

The expression of ERC4, ERD3 and ERD5 variant mRNAs relative to WT-ER mRNA expression within adjacent normal and neoplastic human breast tissues was investigated using previously described semi-quantitative RT-PCR assays (18, 20, 23, 24). These assays allow the determination of the expression of ER-α variant mRNA relative to WT-ER mRNA using a very small amount of starting material, and offer the advantage of allowing investigators to work with histopathologically well characterized human breast tissue regions. One should note however that the sensitivities of the assays used in this study differed from each other. The TP-PCR

assay, previously set up to allow the determination of ERC4 relative expression in tumor samples with very low ER levels, as measured by ligand binding assay, gave a measurable value of expression in 36 out of the 36 samples studied. This contrasts with the detection of 30 out of 36 and 33 out of 36 obtained using ERD3 specific and ERD5 specific primers, respectively. These differences in sensitivity probably result from different primer set efficiencies under our experimental conditions.

A trend towards a higher ERC4 mRNA relative expression in tumor components compared to the normal adjacent tissue component has been observed in the cohort studied (12 out of 18 cases). This difference reached statistical significance when considering the ER positive/PR positive subgroup (n=9, p= 0.019). This result is in agreement with our previous data obtained by comparing ERC4 mRNA expression between independent normal reduction mammoplasty samples and a group of ER positive/PR positive breast tumors (20). The absence of statistically significant differences when considering the total cohort in the present study may result from the low number of matched cases studied or to the different biology of ER negative cases. Further studies are needed to clarify this issue. ERC4 variant mRNA has been previously shown to be more highly expressed in ER positive tumors showing poor prognostic characteristics (presence of more than 4 axillary lymph nodes, tumor size >2 cm, aneuploid, high % S-phase cells) than in ER positive tumor with good prognostic characteristics (absence of axillary lymph node, tumor size < 2 cm, diploid, low % S-phase cells) (25). Moreover, in this previous study, an higher ERC4 mRNA expression has also been observed in ER positive/PR negative tumors, as compared to ER positive/PR positive tumors (25). Interestingly we also have recently reported similar levels of expression of ERC4 mRNA in primary breast tumors and their concurrent axillary lymph node metastases (23). Taken together, these data suggest that the putative role of the ERC4 variant protein might be important in the earliest phases of breast tumorigenesis rather than in the later stages of breast cancer progression. Transient expression assays revealed that the protein encoded by ERC4 mRNA was unable to activate the transcription of an ERE-reporter gene or to modulate the wild-type ER protein activity (19). The biological significance of the changes observed in ERC4 mRNA expression during breast tumorigenesis remains therefore unclear.

A trend towards a higher relative expression of ERD3 mRNA in the normal breast tissue components compared to adjacent neoplastic tissue was found (10 out of 13 cases), which reached statistical significance when the ER positive subgroup was only considered. These data are in agreement with the recently published report of Erenburg et al. who showed a decreased relative expression of ERD3 mRNA in neoplastic breast tissues and

breast cancer compared with independent reduction mammoplasty and breast tumor (21). Transfection experiments performed by these investigators showed that the activation of the transcription of the pS2 gene by estrogen was drastically reduced in the presence of increased ERD3 expression. Moreover, ERD3 transfected MCF-7 cells had a reduced saturation density, exponential growth rate and in vivo invasiveness, as compared to control cells. These data led the authors to hypothesize that the reduction of ERD3 expression could be a pre-requisite for breast carcinogenesis to proceed. They suggested that if high levels of ERD3 could attenuate estrogenic effects in normal breast tissue, low levels might lead to an excessive and unregulated mitogenic action of estrogen.

We observed a significantly higher relative expression of ERD5 mRNA in breast tumor components compared to matched adjacent normal breast tissue. These data confirm our previous observations performed on non-matched normal and neoplastic human breast tissues. Upregulated expression of this variant has already been reported in ER negative/PR positive tumors, as compared to ER positive/PR positive tumor (16, 26), suggesting a possible correlation between ERD5 mRNA expression and breast tumor progression. Interestingly, ERD5 mRNA can be detected in human pituitary adenomas but not in normal pituitary samples (27). This underscores the putative involvment of this ER variant in other tumor systems. Eventhough it has been suggested that ERD5 could be related to the acquisition of insensitivity to antiestrogen treatment (i.e Tamoxifen) (28, 29), accumulating data refute a general role for ERD5 in hormone-resistant tumors (15, 30-32). Only ER positive pS2 positive tamoxifen resistant tumors were shown to express significantly higher levels of ERD5 mRNA, as compared to control tumors (32). Taken together, these data suggest that the exact biological significance of ERD5 variant expression during breast tumorigenesis and breast cancer progression, if any, remains unclear.

Among all the articles published so far on ER variant expression, only a few have investigated expression between matched samples. Recently, Okada et al. reported such a study performed on 15 cases. They observed an apparent difference in ER variant mRNA expression between adjacent normal and tumor samples (33). However, this study was performed using a less sensitive PCR approach, as PCR products were stained using ethidium bromide, and no attempt was made to quantify ER variant mRNA expression relative to WT-ER mRNA expression.

In conclusion, we have shown that the relative expression of ERC4 and ERD5 variant mRNAs was increased in human breast tumor tissue, as compared to normal adjacent tissue, whereas the expression of ERD3 variant

mRNA was decreased in breast tumor tissues. These results, which confirm previous data obtained on independent human breast tissue samples, suggest that the deregulation of the expression of several ER-α variant mRNAs occurs during human breast tumorigenesis. Whether or not a functional role of altered ER-α variant expression is involved in the mechanisms underlying breast tumorigenesis remains to be determined.

References

1. **Green S, Walter P, Kumar V, et al.** 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature. 320:134-139.
2. **Mosselman S, Polman J, Dijkema R.** 1996 ER-beta: identification and characterization of a novel human estrogen receptor. FEBS Letters. 392:49-53.
3. **Evans RM.** 1988 The steroid and thyroid hormone receptor superfamily. Science. 240:889-895.
4. **Kumar V, Green S, Stack G, et al.** 1987 Functional domains of the human estrogen receptor, Cell. 51:941-951.
5. **Ponglikitmongkol M, Green S, Chambon P.** 1988 Genomic organization of the human oestrogen receptor gene. EMBO J. 7:3385-3388.
6. **Enmark E, Pelto-Huikko M, Grandien K, et al.** 1997 Human estrogen receptor β gene structure, chromosomal localization and expression pattern. J Clin Endocrinol Metabol. 82:4258-4265.
7. **Beato M.** 1989 Gene regulation by steroid hormones. Cell. 56:335-344.
8. **Shibata H, Spencer TE, Onate SA, et al.** 1997 Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Rec Prog Horm Res. 52:141-164.
9. **Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH.** 1997 Oestrogen receptor variants and mutations in human breast cancer. Ann Med 29:221-234.
10. **Leygue E, Dotzlaw H, Hare H, Watson PH, Murphy LC.** 1997 Expression of estrogen receptor beta variant mRNAs in human breast tumors. Breast Cancer Res Treat. 46:48, abstract 175.
11. **Vladusic E, Hornby A, Guerra-Vladusic F, Lupu R.** 1998 Expression of estrogen receptor β messenger RNA variant in breast cancer. Cancer Res. 58:210-214.
12. **Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC, Watson PH.** 1998 Estrogen receptor-beta mRNA variants in human and murine tissues. Mol Cell Endocrinol. 138:199-203.
13. **Moore J, McKee D, Slentz-Kesler K, et al.** 1998 Cloning and characterization of human estrogen receptor β isoforms. Biochem Biophys Res Commun. 247:75-78.
14. **Wang Y, Miksicek RJ.** 1991 Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol. 5:1707-1715.

15. **Desai A, Luqmani Y, Coope R, et al.** 1997 Presence of exon 5 deleted oestrogen receptor in human breast cancer: functional analysis and clinical significance. Br J Cancer. 75:1173-1184.

16. **Fuqua SAW, Fitzgerald SD, Chamness GC, et al.** 1991 Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res. 51:105-109.

17. **Chaidarun S, Alexander J.** 1998 A tumor specific truncated estrogen receptor splice variant enhances estrogen stimulated gene expression. Mol Endocrinol, 12:1355-1366.

18. **Leygue ER, Watson PH, Murphy LC.** 1996 Estrogen receptor variants in normal human mammary tissue. J Natl Cancer Inst. 88:284-290.

19. **Dotzlaw H, Alkhalaf M, Murphy LC.** 1992 Characterization of estrogen receptor variant mRNAs from human breast cancers. Mol Endocrinol. 6:773-785.

20. **Leygue E, Murphy LC, Kuttenn F, Watson PH.** 1996 Triple primer polymerase chain reaction. A new way to quantify truncated mRNA expression. Am J Pathol. 148:1097-1103.

21. **Erenburg I, Schachter B, Lopez RM, Ossowski L.** 1997 Loss of an estrogen receptor isoform (ER-alpha deleted 3) in breast cancer and the consequences of its reexpression: interference with estrogen-stimulated properties of malignant transformation. Mol Endocrinol. 11:2004-2015.

22. **Hiller T, Snell L, Watson PH.** 1996 Microdissection/RT-PCR analysis of gene expression. Biotechniques. 21:38-44.

23. **Leygue E, Hall RE, Dotzlaw H, Watson PH, Murphy LC.** Estrogen receptor variant mRNA expression in primary human breast tumors and matched lymph node metastases. Br J Cancer. In press.

24. **Daffada AA, Johnston SR, Smith IE, Detre S, King N, Dowsett M.** 1995 Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. Cancer Res. 55:288-293.

25. **Murphy LC, Hilsenbeck SG, Dotzlaw H, Fuqua SAW.** 1995 Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. Clin Cancer Res. 1:155-159.

26. **McGuire WL, Chamness GC, Fuqua SAW.** 1992 Abnormal estrogen receptor in clinical breast cancer. J Ster Bioch Mol Biol. 43:243-247.

27. **Chaidarun SS, Klibanski A, Alexander JM.** 1997 Tumor specific expression of alternatively spliced estrogen receptor messenger ribonucleic acid variants in human pituitary adenomas. J Clin Endocrinol Metab. 82:1058-1065.

28. **Villa E, Dugani A, Fantoni E, et al.** 1996 Type of estrogen receptor determines response to antiestrogen therapy. Cancer Res. 56:3883-3885.

29. **Klotz DM, Castles CG, Fuqua SAW, Spriggs LL, Hill SM.** 1995 Differential expression of wild-type and variant ER mRNAs by stocks of MCF-7 breast cancer cells may account for differences in estrogen responsiveness. Biochem Biophys Res Commun. 2:609-615.

30. **Rea D, Parker MG.** 1996 Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. Cancer Res. 56:1556-1563.

31. **Madsen MW, Reiter BE, Larsen SS, Briand P, Lykkesfeldt AE.** 1997 Estrogen receptor messenger RNA splice variants are not involved in antiestrogen resistance in sublines of MCF-7 human breast cancer cells. Cancer Res. 57:585-589.

32. **Daffada AA, Johnston SR, Smith IE, Detre S, King N, Dowsett M.** 1995 Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. Cancer Res. 55:288-293.

33. **Okada K, Ichii S, Hatada T, Ishii H, Utsunomiya J.** 1998 Comparison of variant expression of estrogen receptor mRNA in normal breast tissue and breast cancer. Int J Oncol. 12:1025-1028.

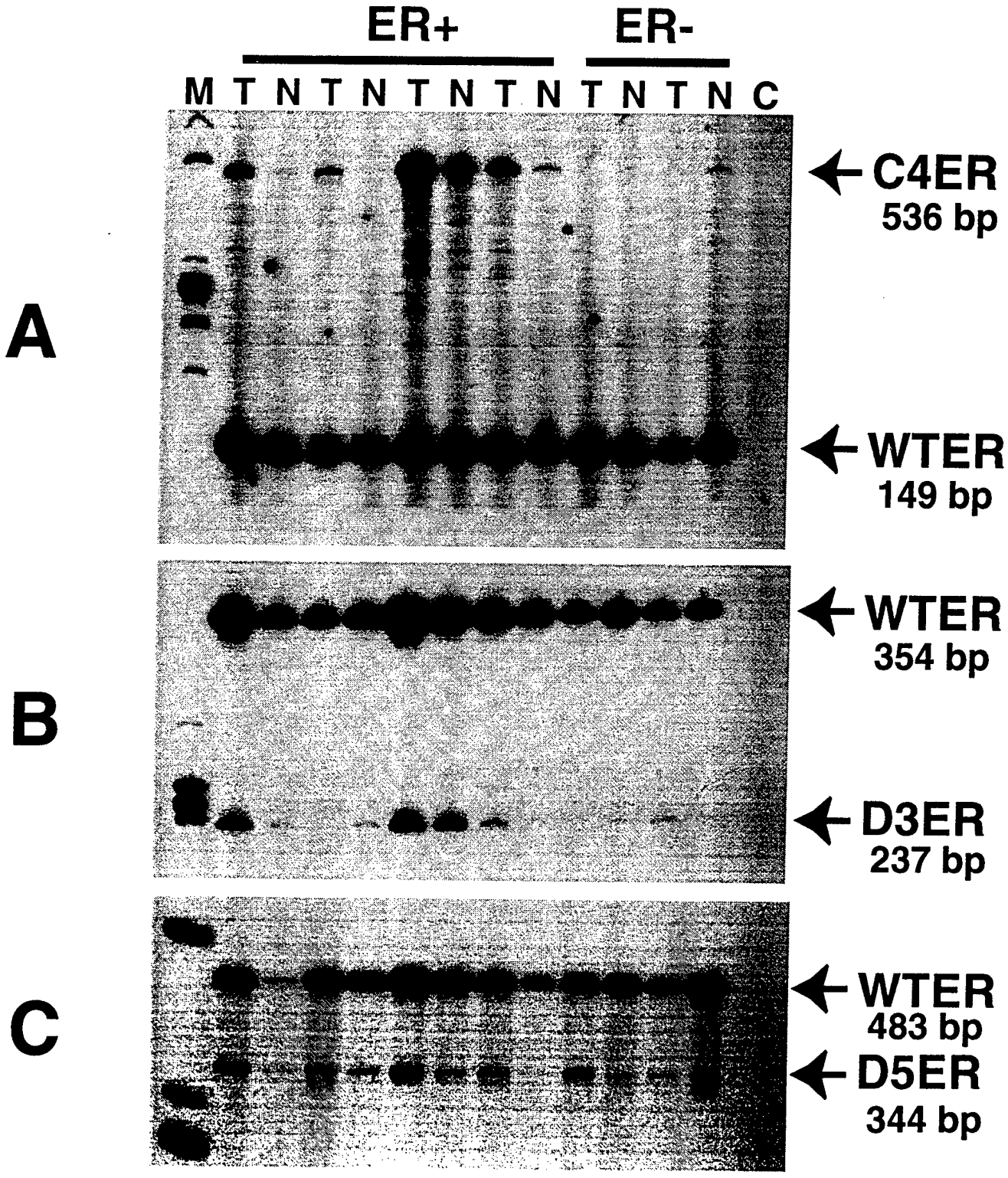
Figures legend

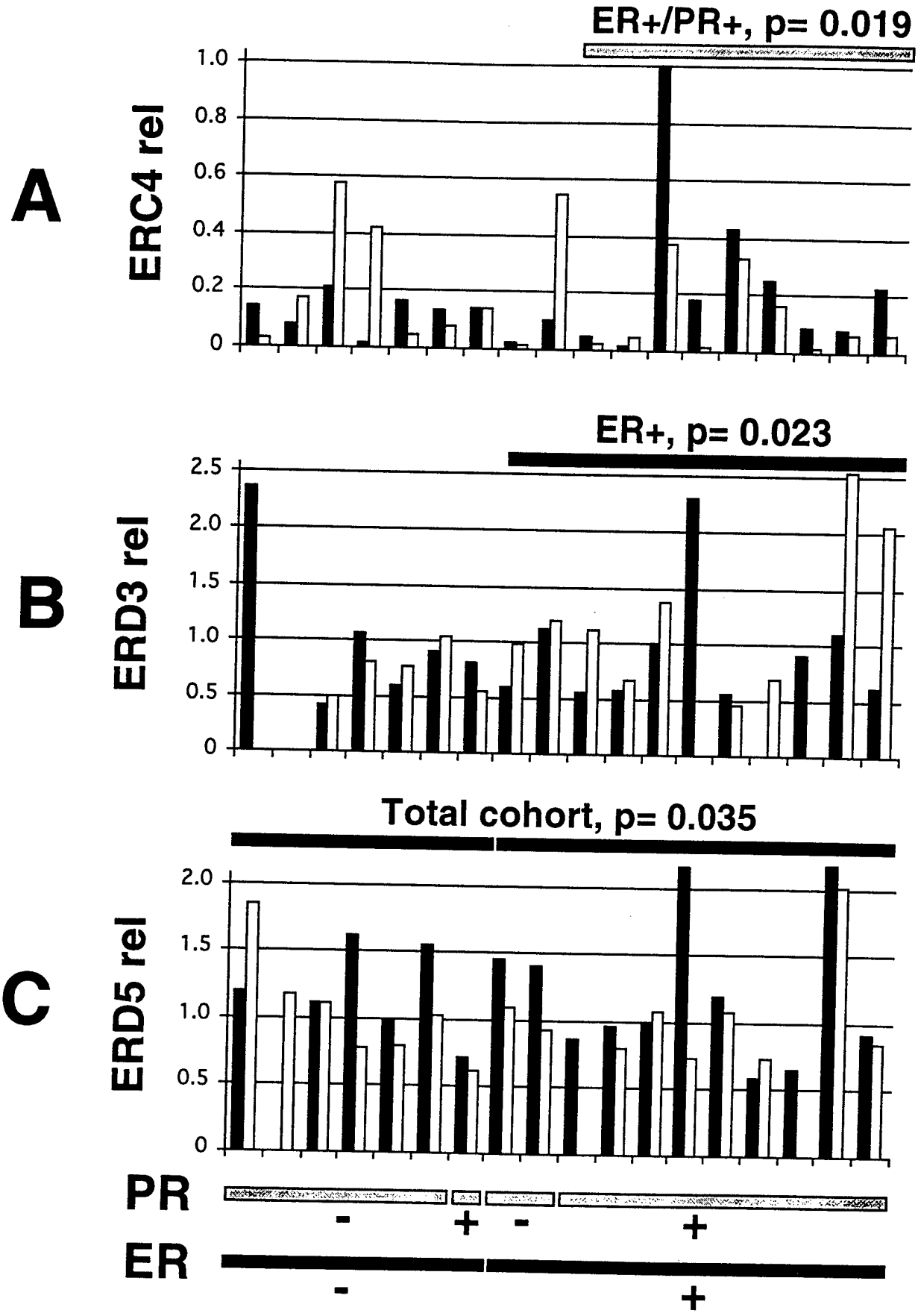
Fig. 1

Detection of ERC4, ERD3 and ERD5 variant mRNAs in matched human breast samples. Total RNA extracted from frozen tissue sections from tumor (T) and adjacent normal (N) breast tissue samples was reverse transcribed and PCR amplified as described in the "Materials and Methods" section : ERU, ERL and C4L primers (**A**), D3U and D3L primers (**B**), and D5U and D5L primers (**C**). Radioactive PCR products were separated on a 6% acrylamide gel and visualized by autoradiography. Bands migrating at 149 bp, 354 bp and 483 bp (**A**, **B**, **C**), 536 bp (**A**), 237 bp (**B**) and 344 bp (**C**) were identified as corresponding to WT-ER (**A**, **B**, **C**), ERC4 (**A**), ERD3 (**B**) and ERD5 (**C**) variant mRNAs, respectively. M: Molecular weight marker (ϕx174 HaeIII digest, Gibco BRL, Grand Island, NY). C, no cDNA added during the PCR reaction.

Fig. 2

Comparison of the relative expression of ERC4, ERD3, and ERD5 variant mRNAs between breast tumor and adjacent matched normal breast samples. Total RNA extracted from tumor (black columns) and matched normal (white columns) breast tissue samples from 18 cases was reverse transcribed and PCR amplified as described in the "Materials and Methods" section using: ERU, ERL and C4L primers (**A**), D3U and D3L primers (**B**), and D5U and D5L primers (**C**). For each case (1-18), signals corresponding to ERC4 (**ERC4 rel**), ERD3 (**ERD3 rel**) and ERD5 (**ERD5 rel**) variant mRNAs were quantified as described in "Materials and Methods" and expressed in arbitrary units for both components. For each sample, the mean of at least three different PCR assays is indicated. Samples not depicted failed to have three measurable signals in the four experiments performed. Cases are sorted by ER status (black bottom lane, negative: - , or positive: +) and PR status (gray bottom lane, negative: - , or positive: +). The significance of the differences between tumor and normal matched components within each subgroup, as tested using the Wilcoxon matched pair test, is indicated when p values are lower than 0.05.


ER+
ER-
M T N T N T N T N T N T N C
A
C4ER
536 bp
WTER
149 bp
B
WTER
354 bp
D3ER
237 bp
C
WTER
483 bp
D5ER
344 bp


A
ER+/PR+, p= 0.019
ERC4 rel
1.0
0.8
0.6
0.4
0.2
0
B
ER+, p= 0.023
ERD3 rel
2.5
2.0
1.5
1.0
0.5
0
C
Total cohort, p= 0.035
ERD5 rel
2.0
1.5
1.0
0.5
0
PR
- + - +
ER
- +

APPENDIX 11.

Submitted to Cancer Research

Estrogen Receptor-β mRNA Expression in Human Breast Tumor Biopsies: Relationship to Steroid Receptor Status and Regulation by Progestins.

Helmut Dotzlaw, Etienne Leygue, Peter H Watson, Leigh C Murphy.

Dept of Biochemistry & Molecular Biology, and Dept of Pathology*, Faculty of Medicine, University of Manitoba, Winnipeg. Manitoba. Canada. R3E 0W3.

Telephone: 204-789-3233

Fax: 204-789-3900

E.mail: lcmurph@cc.umanitoba.ca

Abstract. When the level of estrogen receptor (ER)-β mRNA in tumors, determined by reverse transcription polymerase chain reaction, was assessed according to either ER status or PR status alone, determined by ligand binding assays, the level of ER-β mRNA was significantly lower in PR+ tumors compared to PR - tumors (p=0.036) and no association with ER status was found. Subgroup analysis showed that ER-β mRNA expression in ER+/PR+ breast tumors was significantly less than in ER+/PR- (p=0.009), ER-/PR+ (p=0.029) and ER-/PR- (p=0.023) groups. Interestingly, the ER-β mRNA expression was specifically decreased by progestin in T-47D breast cancer cells. The data suggest the possibility that expression of ER-β in human breast tumors is a marker of endocrine therapy responsiveness.

Introduction.

Both estrogen and antiestrogen can mediate transcriptional activity via the recently identified estrogen receptor-β (ER-β) (1-3). Recently we have shown the presence of ER-β mRNA in both normal and neoplastic human breast tissues (4, 5). Furthermore, the relative expression of ER-α and ER-β mRNA changes between normal human breast tissues and their concurrent matched ER+ breast tumors (6), suggesting that altered expression of ER-α and ER-β occurs and may be functionally involved in breast tumorigenesis. Interestingly, it also appeared that the level of ER-β mRNA varied amongst breast tumors but was not correlated with the expression of ER-α (4), although the two receptor mRNAs were often coexpressed in the same tumor. These observations raised the question of whether the expression of ER-β in breast tumors was correlated with known prognostic and endocrine treatment response markers. In this study the relationship of ER-β mRNA expression to ER and PR status as determined by ligand binding analysis was investigated.

Materials and Methods.

Materials. All cell culture reagents were obtained from Life Technologies (Burlington, Ontario). Medroxyprogesterone acetate (MPA) and dexamethasone were purchased from Sigma (St Louis, Missouri). R5020 and Org 2058 were purchased from Amersham (Oakville, Canada). RU 486 was a gift from Roussel Uclaf (Romainville, France). (α-^{32}P) dCTP was purchased from ICN (Montreal, Que).

Human Breast Tumors.

Forty invasive ductal carcinomas were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected for ER and PR status as determined by ligand binding assays. Ten tumors were classified as ER+/PR+ (ER range 50 - 127 fmol/mg protein, PR range 105 - 285 fmol/mg protein); 10 tumors were classified as ER+/PR- (ER range 59 - 156 fmol/mg protein, PR range 5 - 10 fmol/mg protein); 10 tumors were ER-/PR- (ER range 0 - 2 fmol/mg protein, PR range 0 - 10 fmol/mg protein); 10 tumors were classified as ER-/PR+ (ER range 5 - 9 fmol/mg protein, PR range 51 - 271 fmol/mg protein). These tumors spanned a wide range of grade (grade 4 to 9), determined using the Nottingham grading system.

Cell culture.

T-47D human breast cancer cells were obtained from Dr. D. Edwards (Denver, Colorado). The cells were grown in DMEM supplemented with 5% fetal bovine serum, 100 nM glutamine, 0.3% (v/v) glucose and penicillin/streptomycin as previously described (7). Cells were plated at 1 x 10^6 in 100 mm dishes and 2 days later treated as indicated in the text. The steroids and other compounds were added directly from 1000 x stock solutions in ethanol to

achieve the concentrations indicated. The cells were harvested at the times indicated by scraping with a rubber policeman. After centrifugation the cell pellet was frozen and stored at -70°C until RNA was isolated.

RNA Extraction and RT-PCR conditions.

Total RNA was extracted from 20 µm frozen tissue sections (5 sections per tumor) or frozen cell pellets using Trizol™ reagent (Life Technologies, NY) according to the manufacturer's instructions. One µg of total RNA was reverse transcribed in a final volume of 25 µl as previously described (4).

The primers used consisted of ER-β-U primer (5'-GTCCATCGCCAGTTATCACATC-3'; sense; located in ER–β 130-151) and ER–β–L primer (5'-GCCTTACATCCTTCACACGA-3'; antisense; located in ER–β 371-352). Nucleotide positions given correspond to published sequences of the human ER–β cDNA (2). PCR amplifications were performed and PCR products analyzed as previously described with minor modifications (4). Briefly, 1 µl of reverse transcription mixture was amplified in a final volume of 15 µl, in the presence of 1.5 µCi (α-^{32}P) dCTP (3000 Ci/mmol), 4 ng/µl of ER–β–U/ER–β–L and 0.3 units of Taq DNA polymerase (Life Technologies, NY). Each PCR consisted of 30 cycles (30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (4). PCR products were subcloned and sequenced as previously described (4).

Quantification and Statistical Analysis.

Quantification of signals was carried out by excision of the band corresponding to ER-β cDNA, addition of scintillant and scintillation counting. Three independent PCRs were

performed. In order to control for variations between experiments, a value of 100% was assigned to the case exhibiting the highest signal measured and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified and following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using NIH Image 161/ppc software. Each *GAPDH* signal was also expressed as a percentage of the highest signal observed in the experiment. Two independent PCRs were performed. For each sample, the average of the ER–β signal was then expressed as a percentage of the average *GAPDH* signal. The statistical significance of any differences of the mean ER-β mRNA level between groups was determined using the Mann-Whitney test (two-tailed).

Results.

Measurement of ER–β mRNA expression in primary human breast tumors with different ER and PR status. Previous data have suggested that the level of ER-β mRNA varied widely in human breast tumor samples (4), which raised the question of whether the expression of ER-β in breast tumors was correlated with the known prognostic and treatment response variables, ER and PR status. Four groups, containing 10 breast tumor samples each, were identified according to their ER/PR status as defined by ligand binding analysis (see Materials and Methods). ER-β mRNA levels were measured by RT-PCR and normalized to the GAPDH mRNA level as measured in parallel by RT-PCR. The primers used in this analysis are located in exons 1 and 2 (Fig 1A) of the human ER-β gene (2, 8), and would therefore measure the wild type human ER-β mRNA and all ER-β mRNA variants so far documented (5, 9, 10). Examples of the results obtained are shown in Figure 1B. The results obtained for all tumors

assayed are shown in Figure 1C arranged in groups according to the ER/PR status of the tumor as measured by ligand binding analysis.

The level of ER-β mRNA in ER+/PR+ breast tumors was significantly less than in all other groups (see Table 1), with no significant differences seen amongst the ER+/PR-, ER-/PR+ or ER-/PR- groups. When the level of ER-β mRNA in tumors was assessed according to either ER status or PR status alone, as defined by ligand binding analysis, the level of ER-β mRNA was significantly lower in PR+ tumors compared to PR - tumors (Table 1, G vs H, $p = 0.036$), with no significant differences associated with ER status alone (Table 1, E vs F, $p = 0.323$).

Spearman correlation analysis showed no significant correlations of the level of ER-β mRNA with grade, age, nodal status, or the percentage of normal duct and lobular epithelium, stromal or fat cell content within the tissue section analyzed. However, an inverse relationship was found when the level of ER-β mRNA was correlated with the absolute level of PR as measured by ligand binding analysis ($r = -0.31$, $p = 0.052$), consistent with the data when analyzed using clinically relevant cut-off values for both ER and PR status as shown above.

Regulation of steady state levels of ER-β mRNA by progestins in T-47D human breast cancer cells. The relationship of the level of ER-β mRNA levels with PR status in human breast tumor biopsies suggested the hypothesis that ER-β expression may be regulated by progestins. This hypothesis was investigated using the PR+ T-47D human breast cancer cell line in culture. The steady state level of ER-β mRNA was found to decrease following treatment with 10 nM MPA (Figure 2A). A significant decrease was observed at 6 hours following MPA treatment, and the levels remained decreased for up to 48 hours after

treatment. The effect of MPA on the steady state levels of ER-β mRNA in T-47D cells was first seen with 1 nM of MPA and was maximal between 10 - 100 nM MPA (Figure 2B). The progestin specificity of this response was assessed by treating T-47D cells for 24 hours with MPA, Org 2058, dexamethasone and the antiprogestin RU 486 (Figure 3A & 3B). Both 10 nM MPA and 10 nM of the synthetic progestin Org 2058 significantly decreased the steady state levels of ER-β mRNA, while little if any effect was observed with 100 nM of the synthetic glucocorticoid, dexamethasone. Five hundred nM of the antiprogestin/antiglucocorticoid RU 486 had little if any effect by itself, but inhibited the down-regulation by 10 nM MPA on the level of ER-β mRNA. It was concluded that progestins can down-regulate the steady state levels of ER-β mRNA and that an antiprogestin can inhibit this effect in T-47D human breast cancer cells.

Discussion.

It was previously documented that the level of ER-β mRNA expression in human breast tumors varied widely (4, 8). This raised the question of whether the expression of ER-β in breast tumors was correlated with known prognostic and treatment response markers. The measurement of both estrogen and progesterone receptors in human breast biopsies is routinely used to provide both prognostic and treatment response information (11). Since ER-β is structurally and functionally related to ER-α (1-3, 12) it was relevant to determine if the expression of ER-β was related to the ER and PR status of the tumor as defined by ligand binding assays. Our analysis established that the expression of ER-β mRNA was inversely correlated with PR status generally. Although there was no significant correlation between ER-β mRNA levels and ER status overall, a significant difference in ER-β mRNA levels in those

tumors which were ER+PR+ (lowest expression) and those tumors which were ER-PR+ (higher expression) was observed. This could be interpreted to mean that both ER status and PR status could influence ER-β mRNA expression. However, the differences observed could also be explained by the significant difference in the absolute level of PR expression between the two groups (PR levels determined by ligand binding assays expressed as mean ± SEM, 190 ± 24 fmol/mg protein versus 97 ± 21 fmol/mg proteins, in ER+PR+ and ER-PR+ groups, respectively). This would be consistent with the inverse correlation that was seen with ER-β mRNA and the absolute levels of PR determined by ligand binding analysis, considering all groups together.

These data suggested the possiblity that the expression of ER-β may be regulated by progestins. In T-47D cells which express ER-α, ER-β and PR, the steady state level of ER-β mRNA was specifically decreased by progestin treatment in a time and dose dependent manner. Our data support the hypothesis that the progestin effect is mediated by PR, however, our data do not address whether this occurs via a transcriptional or post-transcriptional mechanism. Interestingly, progestins are known to also decrease the steady state levels of ER-α mRNA and protein in T-47D cells (13). Therefore, PR is able to regulate the expression of both ER-α and ER-β in human breast cancer cells in a similar fashion. However, the interaction of PR and the two distinct ERs is likely to be different. It has been well documented that there is a general positive correlation between ER and PR levels as determined by ligand binding assays in human breast tumors (11). ER status as determined by ligand binding correlates well with both immunological detection of the ER-α protein (14) and ER-α mRNA detection (15). Such data together with other studies (6) suggest that the ER level in breast

tumors as determined by ligand binding in most cases is due to ER-α. Furthermore, ER-β mRNA is the predominant ER mRNA in MDA MB 231 human breast cancer cells (4) and these cells are known to be ER negative by ligand binding assay providing further evidence for the lack of interference of ER-β expression in the determination of ER status by ligand binding assay in the majority of human breast tumors. Interestingly, a significant level of ER-β-like mRNA in human breast cancer cell lines and possibly therefore breast tumors may be represented by exon 8 deleted variants (10) which most likely encode non-estrogen binding ER-β variant proteins, which could not contribute to ER ligand binding assays. Therefore, the available data suggest that the previously observed positive correlation of ER and PR in human breast tumors is due to ER-α expression, underscoring the difference in the relationship of ER-α and ER-β with PR in human breast cancer tissue.

Our data are the first to identify a correlation between ER-β mRNA expression and a known prognostic and treatment response marker in human breast cancer biopsies. The inverse relationship between PR (a good prognostic variable and a marker of response to endocrine therapies) and ER-β suggests that although ER-β is often downregulated in human breast tumors compared to normal human breast tissue (6), its maintainance and/or increased expression in some breast tumors may correlate with a poorer prognosis and the likelihood of failure of response to endocrine therapies such as antiestrogens. This remains to be tested in samples of breast tumors from patients known to have responded or not to have responded to endocrine therapies, in clinical trials. Furthermore, a functional involvement of ER-β in this phenotype remains to be determined. Interestingly, although no agonist activity of tamoxifen-like antiestrogens can be measured through ER-β in a recombinant expression system using

transient transfection and a classical ERE-reporter gene (3), all classes of antiestrogens bound to ER-β result in the transcriptional activation of AP-1 driven reporter genes, again in a transient recombinant model system (12). Since AP-1 regulated genes are often associated with growth and proliferation (16-18) it is tempting to speculate that increased expression of ER-β in human breast tumors could play a role in tamoxifen resistance, in the small number of tumors that appear to proliferate in response to tamoxifen (19, 20).

Acknowledgements. This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Materiel Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). LCM is a Medical Research Council of Canada (MRC) Scientist, PHW is a MRC Clinician-Scientist, EL is a recipient of a USAMRMC Postdoctoral Fellowship. The authors thank Caroline Cumins-Leygue and Helen Bergen for laboratory assistance with the cell culture.

References.

1. Kuiper, G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.-A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci., U.S.A., *93:* 5925-5930, 1996.

2. Mosselman, S., Polman, J., and Dijkema, R. ER-beta: identification and characterization of a novel human estrogen receptor. FEBS Letters, *392:* 49-53, 1996.

3. Tremblay, G., Tremblay, A., Copeland, N., Gilbert, D., Jenkins, N., Labrie, F., and Giguere, V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol. Endocrinol., *11:* 353-365, 1997.

4. Dotzlaw, H., Leygue, E., Watson, P., and Murphy, L. Expression of estrogen receptor-beta in human breast tumors. J. Clin. Endocrinol. Metabol., *82:* 2371-2374., 1997.

5. Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., Murphy, L. C., and Watson, P. H. Estrogen receptor-beta mRNA variants in human and murine tissues. Mol. Cell. Endocrinol., *138:* 199-203, 1998.

6. Leygue, E., Dotzlaw, H., Watson, P., and Murphy, L. Altered estrogen receptor alpha and beta mRNA expression during human breast tumorigenesis. Cancer Res., *58:* 3197-3201, 1998.

7. Alkhalaf, M. and Murphy, L. C. Regulation of c-jun and jun-B by progestins in T-47D human breast cancer cells. Mol. Endocrinol., *6:* 1625-1633, 1992.

8. Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.-A. Human estrogen receptor β gene structure, chromosomal localization and expression pattern. J. Clin. Endocrinol. Metabol., *82:* 4258-4265., 1997.

9. Vladusic, E., Hornby, A., Guerra-Vladusic, F., and Lupu, R. Expression of estrogen receptor β messenger RNA variant in breast cancer. Cancer Res., *58:* 210-214, 1998.

10. Moore, J., McKee, D., Slentz-Kesler, K., Moore, L., Jones, S., Horne, E., Su, J.-L., Kliewer, S., Lehmann, J., and Wilson, T. Cloning and characterization of human estrogen receptor β isoforms. Biochem. Biophys. Res. Commun., *247:* 75-78, 1998.

11. Ravdin, P., Green, S., Dorr, T., McGuire, W., Fabian, C., Pugh, R., Carter, R., Rivkin, S., Borst, J., Belt, R., Metch, B., and Osborne, C. Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: results of a prospective Southwest Oncology Group study. J. Clin. Oncol., *10:* 1284-1291, 1992.

12. Paech, K., Webb, P., Kuiper, G., Nilsson, S., Gustafsson, J., angst, Kushner, P. J., and Scanlan, T. S. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science, *277:* 1508-1510, 1997.

13. Read, L., Greene, G., and Katzenellenbogen, B. Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists and growth factors. Mol. Endocrinol., *3:* 295-304, 1989.

14. McClelland, R., Berger, U., Miller, L., Powles, T., and Coombes, R. Immunocytochemical assay for estrogen receptor in patients with breast cancer: relationship to biochemical assay and outcome of therapy. J. Clin. Oncol., *4:* 1171-1176, 1986.

15. May, E., Mouriesse, H., May-Leven, F., Contesso, G., and Delarue, J. A new approach allowing an early prognosis in breast cancer: the ratio of estrogen receptor (ER) ligand binding activity to the ER-specific mRNA level. Oncogene, *4:* 1037-1042, 1989.

16. Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J. Biol. Chem., *269:* 16433-16442, 1994.

17. Weisz, A. and Bresciani, F. Estrogen regulation of proto-oncogenes coding for nuclear proteins. Crit. Rev. Oncog., *4:* 361-388, 1993.

18. Piechaczyk, M. and Blanchard, J. c-fos protooncogene regulation and function. Crit Rev Oncol. Hematol., *17:* 93-131, 1994.

19. Howell, A., Dodwell, D., Anderson, H., and Redford, J. Response after withdrawal of tamoxifen and progestogens in advanced breast cancer. Ann. Oncol., *3:* 611-617, 1992.

20. Howell, A., Defriend, D., Robertson, J., Blamey, R., and Walton, P. Response to a specific antioestrogen (ICI182780) in tamoxifen resistant breast cancer. Lancet, *345:* 29-30, 1995.

Legends to Figures.

Figure 1.

A. Schematic diagram of the human ER-β cDNA showing the priming sites of the upper and lower primers used for the analysis of ER-β mRNA by RT-PCR.

B. Expression of ER-β mRNA in human breast tumor biopsy samples according to ER and PR status determined by ligand binding assay. The top panel shows an autoradiogram of the RT-PCR assays for ER-β mRNA obtained from representative samples of tumors which were classifed as ER+/PR+, ER+/PR-, ER-/PR+ and ER-/PR- as described in Materials and Methods. The bottom panel shows the ethidium bromide stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples.

C. Quantification of ER–β mRNA expression within human breast tumors classified according to ER and PR status as determined by ligand binding assay. Total RNA, extracted from the tumors, was reverse transcribed, PCR amplified and PCR products were separated on acrylamide gel as described in "Materials and Methods". Signals have been quantified and normalized as indicated in the "Materials and Methods" section. ER+/PR+ tumors are black circles; ER+/PR- tumors are open circles; ER-/PR+ tumors are black squares; ER-/PR- tumors are open squares. The horizonal line represents the median value in each group.

Figure 2.

A. Time dependent downregulation of ER–β mRNA expression in T-47D human breast cancer cells. The top panel shows an autoradiogram of ER–β mRNA levels determined by RT-PCR after treatment with 10 nM MPA for the indicated time periods. The middle panel shows the

the ethidium bromide stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. The bottom panel shows the results presented as a histogram after quantification and normalization of the ER–β signal as described in the Materials and Methods. This experiment was replicated twice.

B. Dose dependent downregulation of ER–β mRNA expression in T-47D human breast cancer cells. The top panel shows an autoradiogram of ER–β mRNA levels determined by RT-PCR after treatment with vehicle alone (V) and varying concentrations of MPA for 24 hours. The middle panel shows the the ethidium bromide stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples. The bottom panel shows the results presented as a histogram after quantification and normalization of the ER–β signal as described in the Materials and Methods.

Figure 3 .

A. Steroid specificity of the downregulation of ER–β mRNA expression in T-47D human breast cancer cells. The top panel shows an autoradiogram of ER–β mRNA levels determined by RT-PCR after 24 hours of treatment with vehicle alone (V), 10 nM MPA, 10 nM Org2058 (ORG), 500 nM RU 486 (RU) and 10 nM MPA + 500 nM Ru 486 (MPA+RU). The middle panel shows the ethidium bromide stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples. The bottom panel shows the results presented as a histogram after quantification and normalization of the ER–β signal as described in the Materials and Methods. This experiment was replicated twice.

B. Steroid specificity of the downregulation of ER–β mRNA expression in T-47D human breast cancer cells. The top panel shows an autoradiogram of ER–β mRNA levels

determined by RT-PCR after 24 hours of treatment with vehicle alone (V), 10 nM MPA, and 10 nM Dexamethasone (DEX). The middle panel shows the ethidium bromide stained gel of the RT-PCR analysis of *GAPDH* mRNA run in parallel for the same samples. The bottom panel shows the results presented as a histogram after quantification and normalization of the ER–β signal as described in the Materials and Methods.

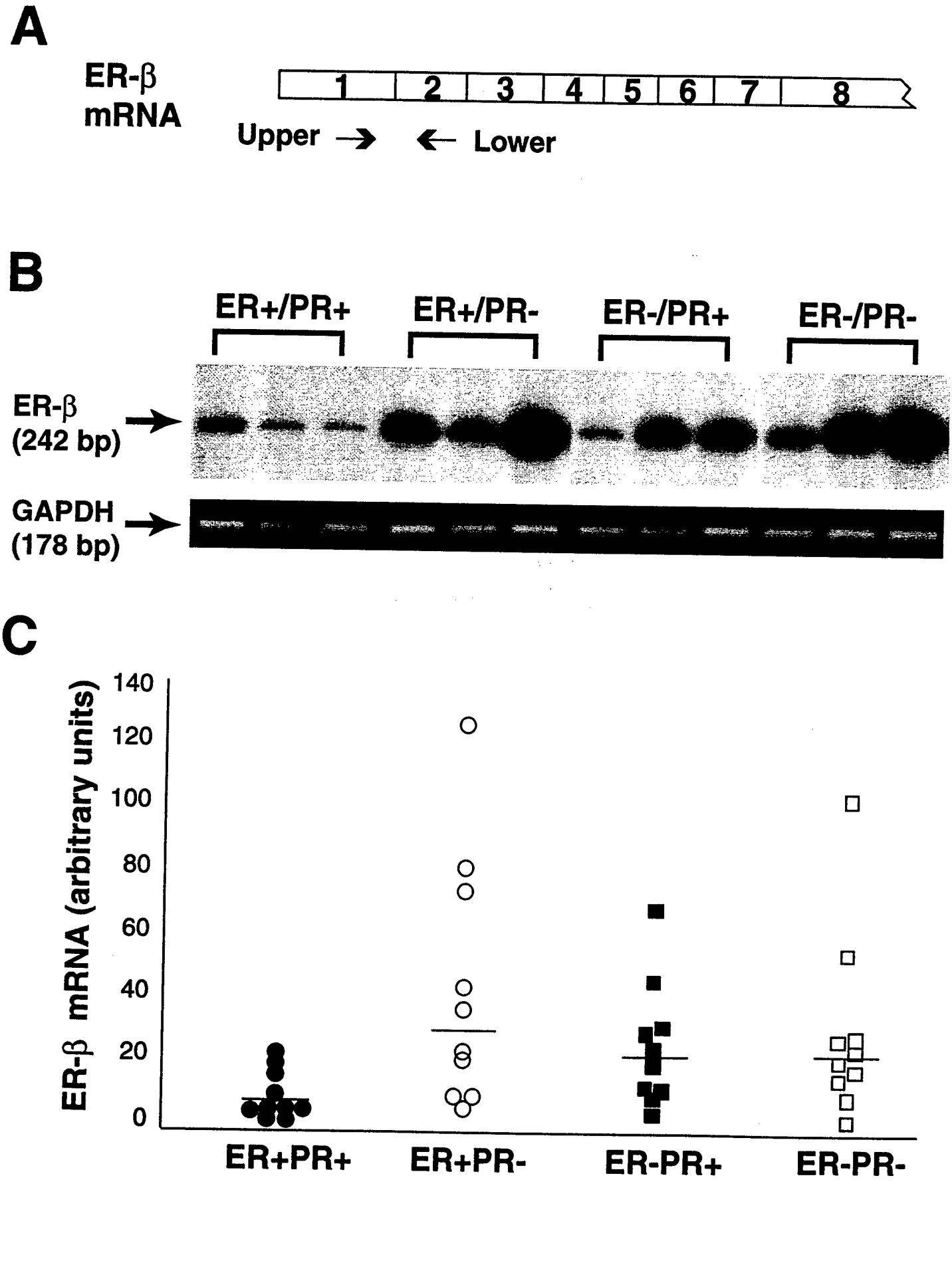
Table 1

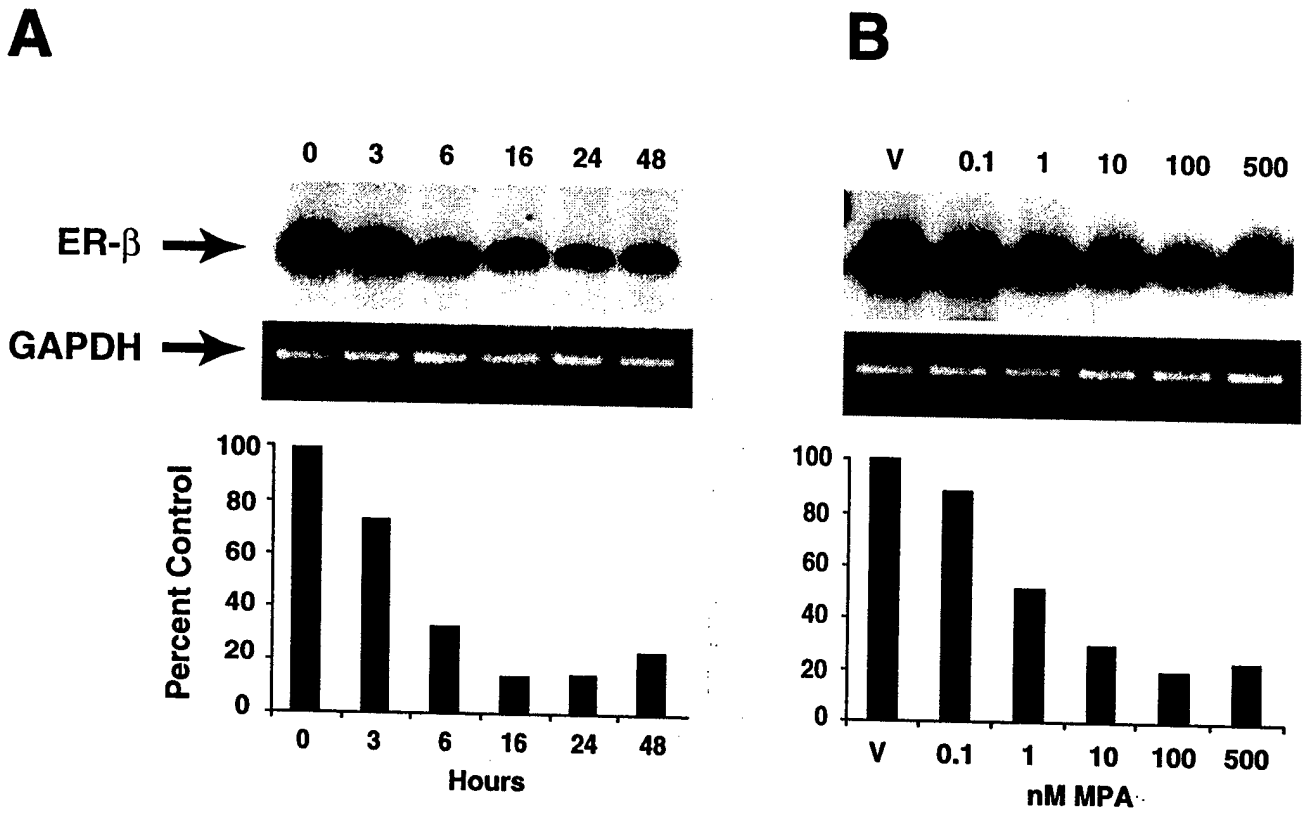
Summary of ER-β mRNA Levels According to Steroid Receptor Status.

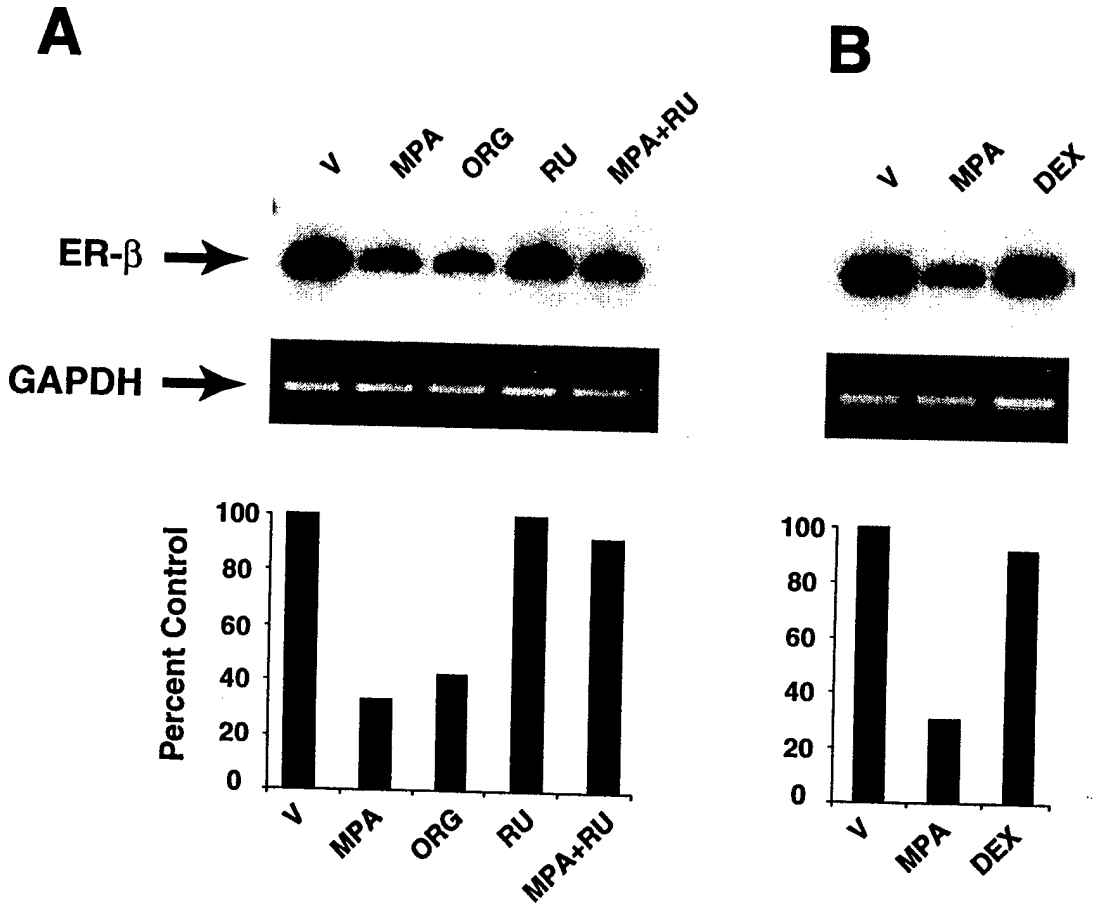
ER/PR Status	Number*	ER-β mRNA Level (mean ± S.E.M.)	Statistical Significance**
A. ER+/PR+	10	11 ± 7.2	
B. ER+/PR-	10	45 ± 12	A vs B, p = 0.009
C. ER-/PR+	10	26 ± 6	A vs C, p = 0.029
D. ER-/PR-	10	31 ± 9.3	A vs D, p = 0.023
E. ER+	20	28 ± 7.2	
F. ER-	20	28 ± 5.4	E vs F, n.s.
G. PR+	20	19 ± 3.5	
H. PR-	20	38 ± 7.7	G vs H, p = 0.036

*, number of tumors per group

**, Mann-Whitney test (two tailed)


A
ER-β mRNA
1
2
3
4
5
6
7
8
Upper
Lower
B
ER+/PR+
ER+/PR-
ER-/PR+
ER-/PR-
ER-β (242 bp)
GAPDH (178 bp)
C
ER-β mRNA (arbitrary units)
140
120
100
80
60
40
20
0
ER+PR+
ER+PR-
ER-PR+
ER-PR-


A
0 3 6 16 24 48
ER-β
GAPDH
Percent Control
100
80
60
40
20
0
0 3 6 16 24 48
Hours
B
V 0.1 1 10 100 500
100
80
60
40
20
0
V 0.1 1 10 100 500
nM MPA


A
V
MPA
ORG
RU
MPA+RU
ER-β
GAPDH
Percent Control
100
80
60
40
20
0
V
MPA
ORG
RU
MPA+RU
B
V
MPA
DEX
100
80
60
40
20
0
V
MPA
DEX

APPENDIX 3

EXPRESSION OF ESTROGEN RECEPTOR BETA 1, BETA 2 AND BETA 5 mRNAs IN HUMAN BREAST TISSUE[1]

Etienne Leygue[2], Helmut Dotzlaw, Peter H. Watson, Leigh C. Murphy

Affiliations of authors: Department of Biochemistry and Molecular Biology [E. L., H. D, L. C. M.] and Department of Pathology [P. H. W.], University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3EOW3.

Running title: ER beta 1, 2 and 5 mRNAs in human breast tissue.

Key words: Estrogen receptor beta, variant, human breast tissue, cancer, PCR, TP-PCR.

Footnotes:

[1] This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Materiel Command (USAMRMC, grant number DAMD17-95-1-5015). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC). P. H. W. is a Medical Research Council of Canada (MRC) Clinician-Scientist, L. C. M. is an MRC Scientist, E. L. is a recipient of a USAMRMC Postdoctoral Fellowship (grant number DAMD17-96-1-6114).

[2] To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, MB. R3E OW3, Canada. Phone: (204) 789 3812; Fax: (204) 789 3900; E-mail:eleygue@cc.umanitoba.ca

[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; TP-PCR, triple primer polymerase chain reaction.

Abstract

A triple primer polymerase chain reaction assay was devloped, based on the co-amplification of estrogen receptor beta 1 (ER–β1), beta 2 (ER–β2) and beta 5 (ER–β5) cDNAs, to investigate the relative expressions of the corresponding mRNAs in breast cancer lines and in 53 independent breast tumors. The expression of ER–β2 and ER–β5 mRNAs was higher than that of ER–β1 mRNA in both cancer cell lines and breast tumors. An increase in the ratio of ER–β2/ER–β1 and ER–β5/ER–β1 mRNA expression was observed, that positively correlated with the level of tumor inflammation and tumor grade. A trend towards an increase of these ratios was also found in tumors, as compared to the normal adjacent breast tissue available for 13 cases. Our data suggest that changes in the relative expression of ER–β1, ER–β2, and ER–β5 mRNAs occur during breast tumorigenesis and tumor progression.

Introduction

Estrogens regulate the growth and the development of normal human mammary tissue and are also involved in breast tumor progression (1). Indeed, estrogens are thought to promote the growth of breast tumors through their mitogenic effects on breast cancer cells. The ability of anti-estrogens such as tamoxifen or raloxifene to inhibit estrogenic action provides the basic rationale for the use of endocrine therapies. Estrogen action is believed to be mediated mainly through two estrogen receptors, estrogen receptor alpha (ER–α, 2) and estrogen receptor beta 1 (ER–β1, 3, 4). These two receptors, that are encoded by two different mRNAs containing eight exons each (5, 6), belong to the steroid/thyroid/retinoic acid receptors super-family (7). ER–α and ER–β1 share the same structural and functional domain composition (8), defined as region A to F (Fig. 1A). The A-B regions contain the N-terminal transactivation function (AF-1) of the receptors, whereas the C region of the molecule contains the DNA binding domain. The ligand binding domain as well as the second transactivation function (AF-2) are located within the E region of the receptors. The receptors, once bound to the ligand, are subject to conformational changes that result in complexes containing dimers of receptors/hormone which recognize estrogen responsive elements located upstream of target genes. Interactions between ERs and accessory proteins ultimately lead to the modification of the transcription of these genes (9). The ER-ligand complexes can also

interact with c-*fos*/c-*jun* complexes to modify the transcription of target genes through AP1 enhancer elements (10, 11). Differential activation of ER–α and ER–β1 by the antiestrogen 4-hydroxytamoxifen, determined by activation of ERE-regulated reporter genes as well as differential activation of AP1-regulated reporter genes by the two ERs have been observed (11, 12). As well, since heterodimerization of ER–α and ER–β1 has also been shown, putative cross-talk between the two signaling pathways is possible (4, 13).

Several variant forms of ER–α and ER–β1 mRNAs have been identified (for a review see 14, 15-17). Among them, exon-deleted variant mRNAs, that would encode ER-like proteins missing some of the functional domains of the wild-type receptors, which could interfere with ER–α and/or ER–β1 signaling pathways. Indeed, exon 5-deleted and exon 7-deleted ER–α variant proteins have been shown, in vitro, to exhibit a constitutive transcriptional (18) and a dominant negative activity (19) on ER–α, respectively. More recently, an ER–β2 variant, deleted of regions encoded by ER–β1 exon 8 sequences, has been shown to heterodimerize with both ER–β1 and ER–α and to inhibit ER–α DNA binding capability (20, 21). The ability of ER–α variants to potentially interfere with the ER–α signaling pathways raised the question of their possible involvement in mechanisms underlying breast tumorigenesis and tumor progression. While many data have been published documenting the differential expression of ER–α variants at different stage of breast cancer progression (14) no studies have been performed comparing the relative expression of ER–β variant mRNAs in human breast tissue. We have developed a triple primer polymerase chain reaction (TP-PCR) assay to evaluate the relative expression of ER–β1, ER–β2, ER–β4 and ER–β5 variant mRNAs. As shown Fig. 1A, ER–β2, ER–β4 and ER–β5 variant mRNAs do not contain exon 8 ER–β1 sequences but share similar 3'end sequences. This assay was used to evaluate the relative expression of ER–β1, ER–β2 and ER–β5 mRNA within breast tumors (n=53) and in some cases (n=13), within adjacent normal breast tissue.

Materials and methods

Human breast tissues and tumor cell lines.

Fifty three cases were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As previously reported, all cases in the Bank have been processed to provide paraffin embedded tissue blocks and mirror image frozen tissue blocks (22). Histopathological analysis was performed on Hematoxylin/Eosin stained

sections from the paraffin tissue block to estimate, for each case, the proportions of tumor and normal epithelial cells, fibroblasts and fat, as well as to determine the levels of inflammation and Notthingam grade scores (23). The age of the patients ranged between 39 and 87 years (n = 53, median = 67). Tumors spanned a wide range of ER (from 0 to 159 fmol/mg protein, n = 53, median = 9 fmol/mg protein) and progesterone receptor (PR values ranging from 0 to 285 fmol/mg protein, n = 53, median = 10 fmol/mg protein), as measured by ligand binding assay. These tumors also covered a wide spectrum of grade (Notthingam grading scores from 1 to 9, n = 47, median 7). Inflammation levels were assessed for 51 cases by scoring the extent of lympho-histocystic infiltrates throughout the section using a semiquantitative scale from 0 (low to minimal infiltration) to 5 (marked infiltrate). For thirteen cases, matched adjacent normal tissue blocks were also available. The characteristics of this subset of 13 tumors were as follow: ER status ranged from 0 to 159 fmol/mg protein (median = 3.5 fmol/mg protein), PR status ranged from 4.9 to 134 fmol/mg protein (median = 8.5), Notthingam grade scores ranged from 5 to 9 (median 7), inflammation levels ranged from 1 to 5 (median 3), and patients were between 39 and 75 years old (median = 54).

MDA-MB-231, MDA-MB-468, ZR-75, BT-20, T-47D, and MCF-7 breast cancer cells were grown and poly-A mRNA was obtained as previously described (24). Total RNA was extracted from frozen breast tissue sections using Trizol™ reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions, and quantified spectrophotometrically. One µg of total RNA was reverse transcribed in a final volume of 25 µl as previously described (25).

Primers and PCR conditions.

The primers used consisted of ER–β1U primer (5'-CGATGCTTTGGTTTGGGTGAT-3'; sense; located in exon 7, position 1400-1420, Genbank accession number AB006590), ER–β1L primer (5'-GCCCTCTTTGCTTTTACTGTC-3'; antisense; located in exon 8, position 1667-1648, Genbank accession number AB006590), and ER–β2L (5'-CTTTAGGCCACCGAGTTGATT-3'; antisense; located in ER–β2 extra-sequences, position 1933-1913, Genbank accession number AF051428). PCR amplifications were performed and PCR products analyzed as previously described with minor modifications (25). Briefly, 1 µl of reverse transcription mixture was amplified in a final volume of 15 µl, in the presence of 1 μCi of [α-^{32}P] dCTP (3000 Ci/mmol), 4 ng/µl of each primer (ER–β1U, ER–β1L and ER–β2L) and 0.3 unit of Taq DNA polymerase

(Gibco BRL, Grand Island, NY). Each PCR consisted of 30 cycles (30 sec at 60°C, 30 sec at 72°C and 30 sec at 94 °C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was performed in parallel and PCR products separated on agarose gels stained with ethidium bromide as previously described (25). Identity of PCR products was confirmed by subcloning and sequencing, as previously reported (25).

Triple primer PCR validation.

The first series of experiments, performed using cDNAs preparation from breast cancer line mRNA, showed that ER–β1, ER–β2 and ER–β5 cDNAs can be co-amplified and led to the production of three PCR products that were subcloned and sequenced as previously described (25). Spiked cDNA preparations containing 1 fg of purified PCR products corresponding to ER–β1 and ER–β5 mRNAs were amplified together with increasing amounts of ER–β2 PCR product (0, 0.2, 0.4, 1, 4 and 8 fg) in a single PCR tube using the three primers (ER–β1U, ER–β1L and ER–β2L), as described above. Similar experiments were performed using constant amounts of ER–β1 and ER–β2, or of ER–β2 and ER–β5, with increasing amounts of ER–β5 or ER–β1 PCR products, respectively. In parallel, preparations containing 1 fg of each PCR products alone were also amplified. In every case, PCR products were separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and autoradiographed. Signals were quantified by excision of the appropriate bands and counting in a scintillation counter (Beckman). For each sample, ER–β1, ER–β2 and ER–β5 signals were expressed as a percentage of the sum of all signals measured (ER–β1 plus ER–β2 plus ER–β5 signals). Experiments have been performed in duplicate and the mean of the relative signals calculated. For each ER–β isoform, regression analyses between the relative signal obtained and the relative initial input (i.e ER–β isoform input expressed as a percentage of ER–β1 plus ER–β2 plus ER–β5 inputs) were performed using GraphPad Prism^R software.

Quantification and statistical analyses.

In order to quantitate the relative expressions of ER–β1, ER–β2 and ER–β5 mRNAs within each breast tissue sample, we used the TP-PCR described above. Quantification of ER–β1, ER–β2 and ER–β5 signals was

carried out by excision of the bands and scintillation counting. For each sample, ER–β1, ER–β2 and ER–β5 signals were expressed as a percentage of the sum of all signals measured (ER–β1 plus ER–β2 plus ER–β5 signals). Three independent PCRs were performed and the mean of the relative expressions was calculated. Differences between ER–β1, ER–β2 and ER–β5 relative expression within the cohort studied were tested using the Wilcoxon signed rank test (Two tailed). Correlations with tumor characteristics were tested by calculation of the Spearman coefficient (r).

Results

Validation of the triple primer PCR (TP-PCR) as an approach to evaluate the relative expression of ER–β1, ER–β2 and ER–β5 mRNAs.

We have previously established that TP-PCR provided a reliable method to investigate the expression of a truncated mRNA relative to the wild-type mRNA expression within small breast tissue samples (25). In its initial design, the TP-PCR assay relied on the co-amplification of one truncated and a wild-type cDNA molecule using three primers in the PCR. The upper primer recognized both sequences whereas the two lower primers recognized the variant and the wild-type sequences, respectively. We have shown that the final ratio between the two co-amplified products was linearly related to the initial cDNA input (25).

As shown Fig. 1A, ER–β1, ER–β2, ER–β4 and ER–β5 mRNAs all have exon 7 sequences, but differ from each other in the following sequences. Interestingly, comparison of the sequences revealed that ER–β2, ER–β4 and ER–β5 mRNAs have sequence similarities within their 3' extremities. Therefore, it was possible to use TP-PCR to investigate the relative expression of these variants. Three primers were designed (ER–β1U, ER–β1L and ER–β2L) which recognized exon 7 sequences common to all transcripts, ER–β1 exon 8 specific sequences, and sequences shared by ER–β2, ER–β4 and ER–β5 mRNAs, respectively (Fig. 1A). As shown in Fig. 1A, the expected PCR products resulting from the co-amplification of the corresponding cDNAs are different in size and can be easily distinguished on an acrylamide gel.

The assay was used initially to determine the expression of ER–β1, ER–β2, ER–β4 and ER–β5 mRNAs in several different human breast cancer cell lines (Fig. 1B). Three bands, migrating at an apparent size of 268 bp, 214 bp and 295 bp were observed in all samples. Subcloning and sequencing these bands confirmed their

identity as ER–β1, ER–β2 and ER–β5 cDNAs, respectively (data not shown). We were unable to detect a product of 529 bp which would correspond to the ER–β4 PCR product. Interestingly, in all tumor cell lines, the ER–β1 signal was lower than the ER–β2 and/or ER–β5 signals (Fig. 1B).

Because TP-PCR performed using these primers produced three PCR products, instead of two PCR products obtained in the original validation studies published (25), it was necessary to establish the quantitative relationship between the signals obtained and the initial target concentrations. In order to address this issue, spiked DNA preparations containing equal amounts of ER–β1 and ER–β5 PCR products and increasing amounts of ER–β2 PCR products were amplified (Fig. 2A). In parallel, The relative signals of ER–β1, ER–β2 and ER–β5 have been measured and expressed as a percentage of the sum of the signals measured, as described in the Materials and Methods section. As expected, in the absence of ER–β2 only two bands, corresponding to ER–β1 and ER–β5 PCR products, are observed. The relative signals of ER–β1 and ER–β5 decreased whereas the ER–β2 relative signal increased linearly with increasing ER–β2 input. Indeed, for each ER–β isoform, regression analysis showed a linear correlation between the relative signal of the PCR product measured and its relative input (Fig. 2B). Similar results were obtained when experiments were performed using constant amounts of ER–β1 and ER–β2 with increasing amounts of ER–β5 PCR products, or using constant amounts of ER–β2 and ER–β5 with increasing amounts of ER–β1 (data not shown). It should be noted that the amplification of similar amounts of the three molecules led to the production of three bands of similar intensities (Fig. 2A). It should also be stressed that the ratio ER–β5/ER–β1 was not affected by increasing amounts of ER–β2, and that the ratios ER–β2/ER–β5 and ER–β5/ER–β2 varied as a linear function of the initial ER–β2/ER–β5 and ER–β5/ER–β2 input ratios, respectively (data not shown). We concluded that the TP-PCR assay, performed under the described conditions, provided a reliable method with which to compare breast tissue samples for their relative expression of ER–β1, ER–β2 and ER–β5 mRNAs.

Comparison of the relative expression of ER–β1, ER–β2 and ER–β5 mRNAs in breast tumor tissues.

To determine if alterations occur in the balance between ER–β1, ER–β2 and ER–β5 mRNAs during breast tumor progression, the relative expression of these transcriptss was measured in primary breast tumor tissues from 53 different patients, using the TP-PCR assay described above. These tumors presented a wide spectrum of ER and PR status, as determined by ligand binding assay, and also spanned a wide range of grade and

inflammation levels (for a more detailed description of the cohort characteristics, see the Materials and Methods section). Total RNA was extracted from frozen tissue sections and reverse transcribed as described in Material and Methods. TP-PCR was then performed. Examples of the results obtained are shown in Fig. 3A. Three PCR products migrating at an apparent size of 268 bp, 214 bp and 295 bp were obtained. These PCR products were shown by cloning and sequencing to correspond to ER–β1, ER–β2 and ER–β5 cDNA, respectively. As in our preliminary study performed in human breast cancer cell lines, no band of 529 bp was detected, which would correspond to ER–β4 PCR product. Amplification of the ubiquitously expressed *GAPDH* cDNA, performed to check the integrity of each cDNA studied, revealed similar amounts of cDNA in all samples (data not shown). ER–β1, ER–β2 and ER–β5 signals obtained in 3 independent TP-PCRs were quantified as described in the Materials and Methods section. For each sample, the percentage of each band relative to the sum of the signals obtained has been calculated. The medians of ER–β1, ER–β2 and ER–β5 relative expressions within tumors, sorted according to their grade or to the level of inflammation are presented Fig. 3B. The ER–β1 relative signal was found to be significantly lower than ER–β2 (Wilcoxon sign rank test, $n = 53$, $p = 0.0002$) and ER–β5 (Wilcoxon sign rank test, $n = 53$, $p = 0.004$) signals. A trend towards a higher expression of ER–β2 as compared to ER–β5 was also observed, but did not reach statistical significance (Wilcoxon sign rank test, $n = 53$, $p = 0.09$).

Possible associations between ER–β1, ER–β2 or ER–β5 signals and tumor characteristics were then investigated. ER–β1 relative expression was found (Fig. 3B) to be inversely related to the grade of the tumor ($n = 47$, Spearman $r = -0.33$, $p = 0.02$) and the level of inflammation ($n = 51$, Spearman $r = -0.28$, $p = 0.04$). No other associations were found between ER–β1 expression and ER status, PR status, or age of the patients. ER–β2 mRNA expression increased significantly with the levels of inflammation ($n = 52$, Spearman $r = 0.28$, $p = 0.04$). No other associations were found between ER–β2 and ER–β5 and other characteristics.

Because the ratio between two signals was related to the respective proportion of the two corresponding cDNAs, we also addressed the question of the expression of ER–β2 and ER–β5 relative to ER–β1. The medians of the ratios ER–β2/ER–β1, ER–β5/ER–β1 and ER–β5/ER–β2 within tumors, sorted according to their grade or to the level of inflammation are presented Fig. 3C. ER–β5 and ER–β2 expressions relative to ER–β1 were found positively associated with the tumor grade ($n = 47$; Spearman $r = 0.29$, $p = 0.04$; and Spearman $r = 0.28$, $p =$

0.05, respectively). In addition, one should note that ER–β2 expression relative to ER–β1 expression correlated (n = 52, Spearman r = 0.34, p = 0.01) with levels of inflammation. ER–β2 and ER–β5 expression relative to each other did not correlate with grade, degree of inflammation or any other tumor characteristics.

No correlations were found between the content of the tissue sections analyzed, i.e percentage of normal cells, tumor cells, fibroblasts or fat, and ER–β1, ER–β2 and ER–β5 mRNA relative expression (data not shown).

ER–β1, ER–β2 and ER–β5 mRNAs expression within matched normal and tumor compartments.

To determine whether changes in the expressions of ER–β1, ER–β2 and ER–β5 mRNAs occur during breast tumorigenesis, we compared the relative expression of these transcripts between normal breast tissue and matched adjacent tumors. Normal adjacent breast tissue was available for 13 cases belonging to the cohort described earlier in the text. The characteristics of this tumor subset are detailed in the Materials and Methods section. Total RNA was extracted and following reverse transcription, TP-PCR was performed as described in the Material and Methods section. Typical results are shown Fig. 4A. Quantification of the signals was performed as described earlier in the text. Fig. 4B shows the relative expression within tumor and adjacent normal breast tissues of ER–β1 mRNA. A trend towards a lower ER–β1 signal (9 out of 13 cases, Wilcoxon sign rank test, p=0.06) in the tumor compartment compared to the normal adjacent components was observed. In contrast, trends towards higher expression of ER–β2 (Fig. 4C) and ER–β5 (Fig. 4D) mRNAs relative to ER–β1 mRNA were observed in tumor compartments (8 out of 13 cases, Wilcoxon sign rank test, p=0.10; and 9 out of 13 cases, Wilcoxon sign rank test, p=0.06, respectively).

Discussion

In order to evaluate the relative expression of ER–β1, ER–β2 and ER–β5 mRNAs within small frozen sections of human breast tissues, we have developed an assay based on the co-amplification of the corresponding cDNAS in a single tube, using three primers in the PCR. The quantitative aspect of this assay was validated using preparations containing known amounts of target cDNA. The TP-PCR approach appeared to be a reliable approach to estimate not only the relative expression of each variant within the population of ER–β molecules measured (ie ER–β1, ER–β2 and ER–β5 mRNAs), but also the proportion of each RNA relative to one another. One should note that the set of primers used would detect ER–β4 variant mRNA. However, this variant was not

detected in any breast sample or tumor cell line studied. This might result from either a lower efficiency of amplification of this specific variant in our PCR conditions, or to a lower relative expression of ER–β4 mRNA as compared to ER–β1, ER–β2 and ER–β5. A low expression of ER–β4 mRNA would be consistent with data obtained on breast cancer cell lines by Moore et al. (20).

Our data show that ER–β1, ER–β2 and ER–β5 mRNAs are co-expressed in human breast cancer cells grown in culture. These data confirm the previous observation of Moore et al. (20). These authors however, did not address the question of the relative expression of these mRNAs. Striking differences in the pattern of expression of ER–β1, ER–β2 and ER–β5 mRNAs were found between tumor cell lines. If these differences in expression are conserved at the protein level, one might hypothesize that ER–β signaling pathways, that likely result from the balance between the different forms, vary in these cells. To date, multiple ER–β-like mRNAs have been described, that could encode different proteins that would be difficult to distinguish from each other by Western blot analysis. For example, ER–β1 protein (5) and ER–β2 protein (20) present theoretical molecular masses of 54.2 kDa and 55.5 kDa, respectively. Most likely, antibodies recognizing specifically the different ER–β proteins would be the best approach to address the question of the relative expression of ER–β proteins within breast cancer cells. Higher ER–β2 and ER–β5 expression as compared to ER–β1 expression were observed in cancer cell lines. This suggests that the respective participation of ER–β2 and ER–β5 variants in ER–β signaling pathways within breast cancer cells might be as important as that of ER–β1.

As observed in breast cancer cell lines, ER–β1, ER–β2 and ER–β5 mRNAs were detected in human breast tumors. Consistent with the observations in breast cancer cell lines, ER–β2 and ER–β5 mRNAs were more highly expressed than ER–β1 mRNA in these tissues. However, even though this observation may result directly from the expression of different ER–β isoforms in breast cancer cells, it may also be a consequence of the heterogeneity of the cell populations expressing ER–β molecules, and present in different proportions within the tumor sample analyzed. Indeed, because lymphocytes have previously been shown to express ER–β1, ER–β2 and ER–β5 mRNAs, one could speculate that infiltrating lymphocytes within the tumor might contribute to the higher level of ER–β2 mRNA expression in tumors with higher inflammation levels. Techniques such as *in situ* hybridization or immunocytochemistry, designed to distinguish between the different ER–β isoforms, are needed to address the question of the cellular origin of ER–β isoform expressions *in vivo*.

We observed an inverse relationship between the relative expression of ER–β1 mRNA and tumor grade. It has been shown that the Notthingham grade provides a useful marker of the length of the rdisease free interval and overall survival (23). We have also observed a decrease of the relative expression of ER–β1 in tumor versus normal adjacent components. Taken together, these data suggest that changes in the relative expression of ER–β1, ER–β2 and ER–β5 mRNAs occur during breast tumorigenesis and tumor progression. Whether these changes are a cause or a consequence of tumorigenesis remains to be elucidated.

In conclusion, we have developed a TP-PCR assay allowing the investigation of the relative expressions of ER–β1, ER–β2, and ER–β5 mRNA in human breast tissues. In these tissues, ER–β1 mRNA appeared to have the lowest level of expression when compared to the two other isoforms detected. We found that the relative expression of ER–β1 was inversely related to the grade of the tumor, suggesting that it could be used as a marker of tumor progression. Moreover, a lower relative expression of ER–β1 was observed in tumor versus adjacent normal breast tissues, suggesting that changes in the expression of ER–β isoforms occur during breast tumorigenesis. The cellular origin of the expression of ER–β1, ER–β2 and ER–β5 in breast tumor tissue in vivo remains to be determined as does the putative role of the different ER–β variant forms in the mechanisms underlying tumorigenesis and tumor progression.

References

1 Vorherr, H. (ed.). Breast cancer. Urban and Schwarzenberg press, Baltimore. 1980.

2 Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., and Chambon, P. Human oestrogen receptor cDNA: sequence, expression and homology to v- erb-A. Nature, *320:* 134-139, 1986.

3 Mosselman, S., Polman, J., and Dijkema, R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett., *392:* 49-53, 1996.

4 Ogawa, S., Inoue, S., Watanabe, T., Hiroi, H., Orimo, A., Hosoi, T., Ouchi, Y, and Muramatsu, M. The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. Biochem. Biophys. Res. Commun., *243:* 129-132, 1998.

5 Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J. A. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J. Clin. Endocrinol. Metab., *82:* 4258-4265, 1997.

6 Ponglikitmongkol, M. Green, S., and Chambon, P. Genomic organization of the human oestrogen receptor gene. Embo J., *7:* 3385-3388, 1988.

7 Evans, R. M. The steroid and thyroid receptor super-family. Science, *240:* 889-895, 1988.

8 Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R., and Chambon, P. Functional domains of the human estrogen receptor. Cell, *51:* 941-951, 1987.

9 Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog. Horm. Res., *52:* 141-164, 1997.

10 Umayahara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J. Biol. Chem., *269:* 16433-16442, 1994.

11 Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science, *277:* 1508-1510, 1997.

12 Watanabe, T., Inoue, S., Ogawa, S., Ishii, Y., Hiroi, H., Ikeda, K., Orimo, A., and Muramatsu, M. Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context, and estrogen receptor subtype: functional difference between estrogen receptors alpha and beta. Biochem. Biophys. Res. Commun., *236:* 140-145, 1997.

13 Cowley, S. M., Hoare, S., Mosselman, S., and Parker, M. G. Estrogen receptors alpha and beta form heterodimers on DNA. J. Biol. Chem., *272:* 19858-19862, 1997.

14 Murphy, L. C., Leygue, E., Dotzlaw, H., Douglas, D., Coutts, A. and Watson, P. H. Estrogen receptor varaints and mutations. Ann. Med., *29:* 221-224, 1997.

15 Vladusic, E. A., Hornby, A. E., Guerra-Vladusic, F. K., and Lupu, R. Expression of estrogen receptor beta messenger RNA variant in breast cancer. Cancer Res., *58:* 210-214, 1998.

16 Lu, B., Leygue, E., Dotzlaw, H., Murphy, L. J., Murphy, L. C., and Watson, P. H. Estrogen receptor-b mRNA variants in human and murine tissues. Mol. Cell. Endocrinol., *138:* 199-203, 1998.

17 Shupnik, M. A., Pitt, L. K., Soh, A. Y., Anderson, A., Lopes, M. B., and Laws Jr., E. R. Selective expression of estrogen receptor α and β isoforms in humn pituirary tumors. J. Clin. Endocrinol. Metab., *83:* 3965-3972, 1998.

18 Fuqua, S. A. W., Fitzgerald, S. D., Chamness, G. C., Tandon, A. K., McDonnell, D. P., Nawaz, Z., O'Malley, B. W., and McGuire, W. L. Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res., *51:* 105-109, 1991.

19 Wang, Y., and Miksicek, R. J. Identification of a dominant negative form of the human estrogen receptor. Mol. Endocrinol., *5:* 1707-1715, 1991.

20 Moore, J. T., McKee, D. D., Slentz-Kesler, K., Moore, L. B., Jones, S. A., Horne, E. L., Su, J. L., Kliewer, S. A., Lehman, J. M., and Willson, T. M. Cloning and characterization of human estrogen receptor beta isoforms. Biochem. Biophys. Res. Commun., *247:* 75-78, 1998.

21 Ogawa, S., Inoue, S., Watanabe, T., Hiroi, H., Orimo, A., Hosoi, T., Ouchi, Y, and Muramatsu, M. Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor of estrogen action in human. Nucleic Acids Res., *26:* 3505-3512, 1998.

22 Hiller, T., Snell, L., and Watson, P. H. Microdissection RT-PCR analysis of gene expression in pathologically defined frozen tissue sections. Biotechniques, *21:* 38-44, 1996.

23 Elston, C. W., and Ellis, I. O. Pathological prognostic factors in breast cancer. Histopathology, *19:* 403-410, 1991.

24 Dotzlaw, H., Alkhalaf, M., and Murphy, L. C. Characterization of estrogen receptor variant mRNAs from human breast cancers. Mol. Endocrinol., *6:* 773-785, 1992.

25 Leygue, E., Murphy, L. C., Kuttenn, F., and Watson, P. H. Triple primer PCR: A new way to quantify truncated mRNA expression. Am. J. Pathol., *148:* 1097-1103, 1996.

Figures legend

Fig. 1

Presentation of the triple primer polymerase chain reaction (TP-PCR) assay and relative expressions of ER–β1, ER–β2, ER–β4 and ER–β5 mRNAs in breast cancer cell lines. A: The structural and functional domains (AF) of the ER–β1 protein (AF-1: Transactivation function 1, DBD and LBD: DNA and ligand binding domains, respectively) are shown together with the corresponding exonic structure (exons 1 to 8) of the ER–β1 mRNA. For each cDNA (β1, β2, β4 and β5), common sequences and specific sequences are depicted by white and gray boxes, respectively. ER–β1U, ER–β1L and ER–β2L primer annealing sites are represented by hatched, dotted and black boxes, respectively. The sizes of the possible PCR products (black bars) obtained after TP-PCR are indicated. **B:** Breast cancer cell line (MDA MB 231, MDA MB 468, ZR75, BT 20 T-47D and MCF 7) poly-A mRNAs have been reverse transcribed, TP-PCR performed and PCR products separated on an acrylamide gel as described in the Materials and Methods section. PCR products migrating at an apparent size of 295 bp, 268 bp and 214 bp have been subcloned, sequenced, and identified as corresponding to ER–β5, ER–β1 and ER–β2 mRNAs, respectively. M: molecular size markers (ϕx174 RF DNA/Hae III fragments, Gibco BRL, Grand Island, NY).

Fig. 2

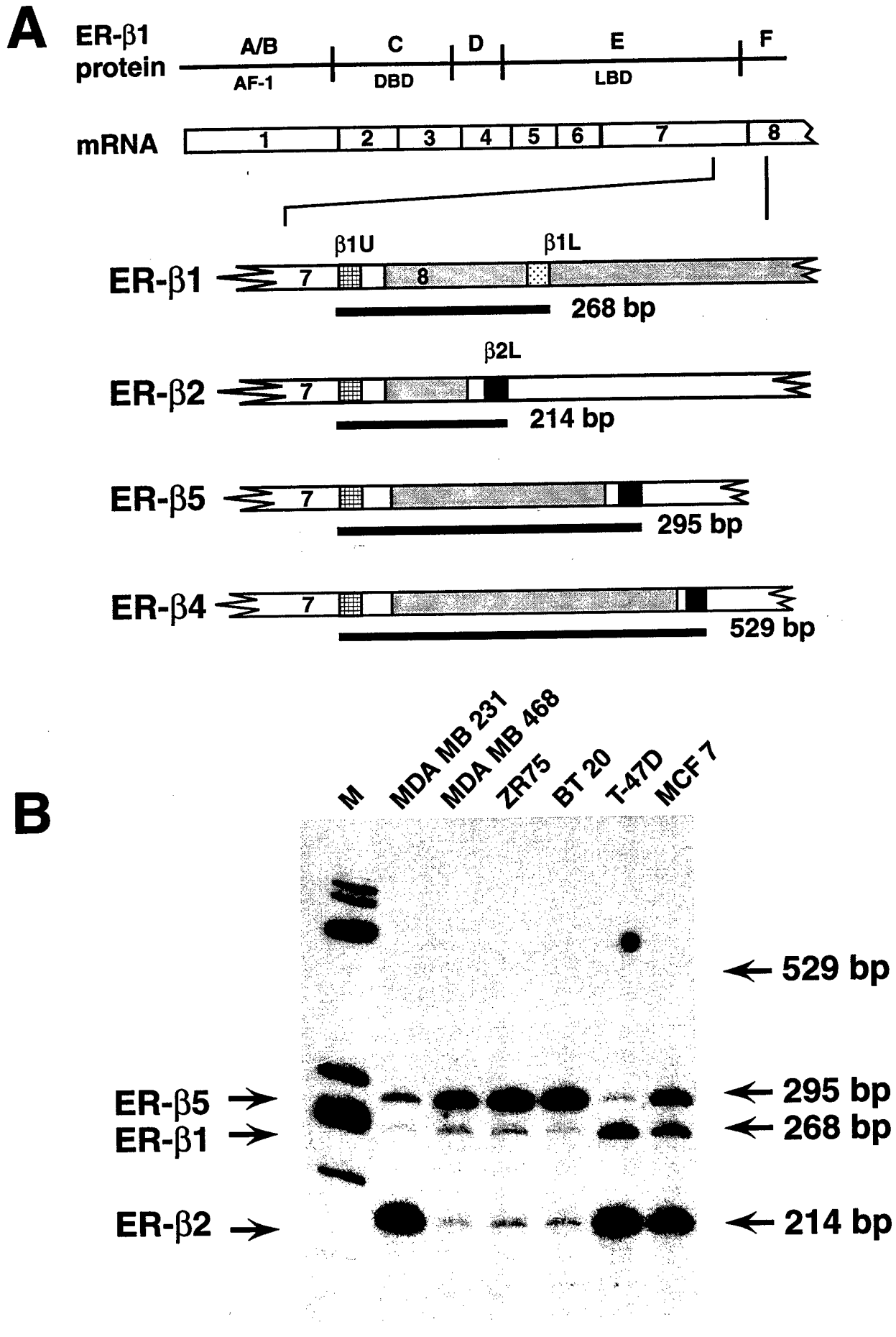
TP-PCR validation. A: Spiked DNA preparations, containing various amounts (indicated in fg above the autoradiogram) of ER–β5, ER–β1 and ER–β2 purified PCR products (β5, β1, and β2) were amplified by TP-PCR, and PCR products separated on an acrylamide gel as specified in the Materials and Methods section. The autoradiogram shows the PCR products obtained. **B:** Signals corresponding to ER–β5, ER–β1 and ER–β2 PCR products have been quantified in each lane as described in the Materials and Methods section. For each ER–β isoform, the relative signal observed (% signal, expressed as a percentage of the sum β1 plus β2 plus β5 signals) is presented as a function of the initial relative cDNA input (%input, expressed as a percentage of the sum β1 plus β2 plus β5 inputs). The regression coefficients (r) and the p value of the association are also presented.

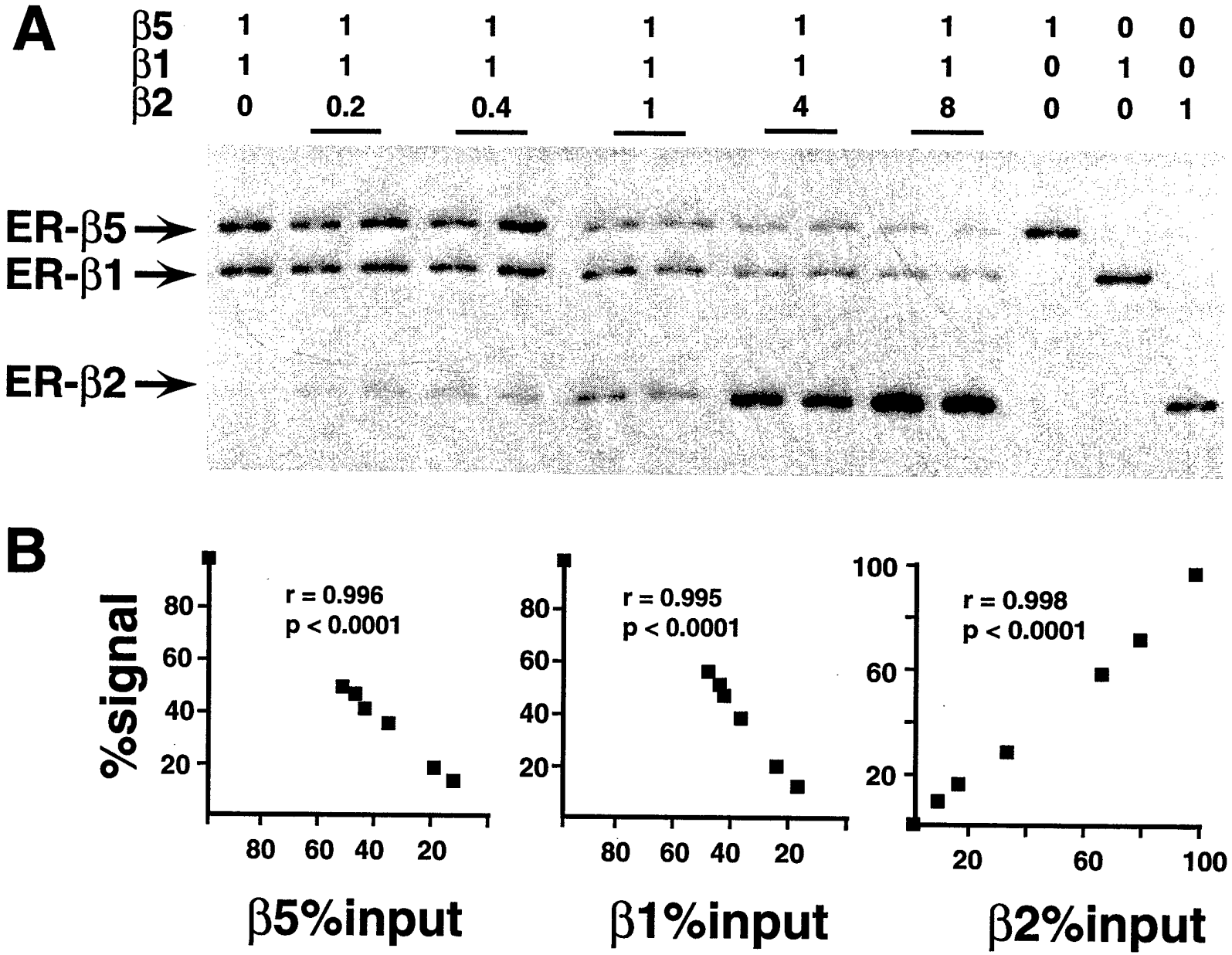
Fig. 3

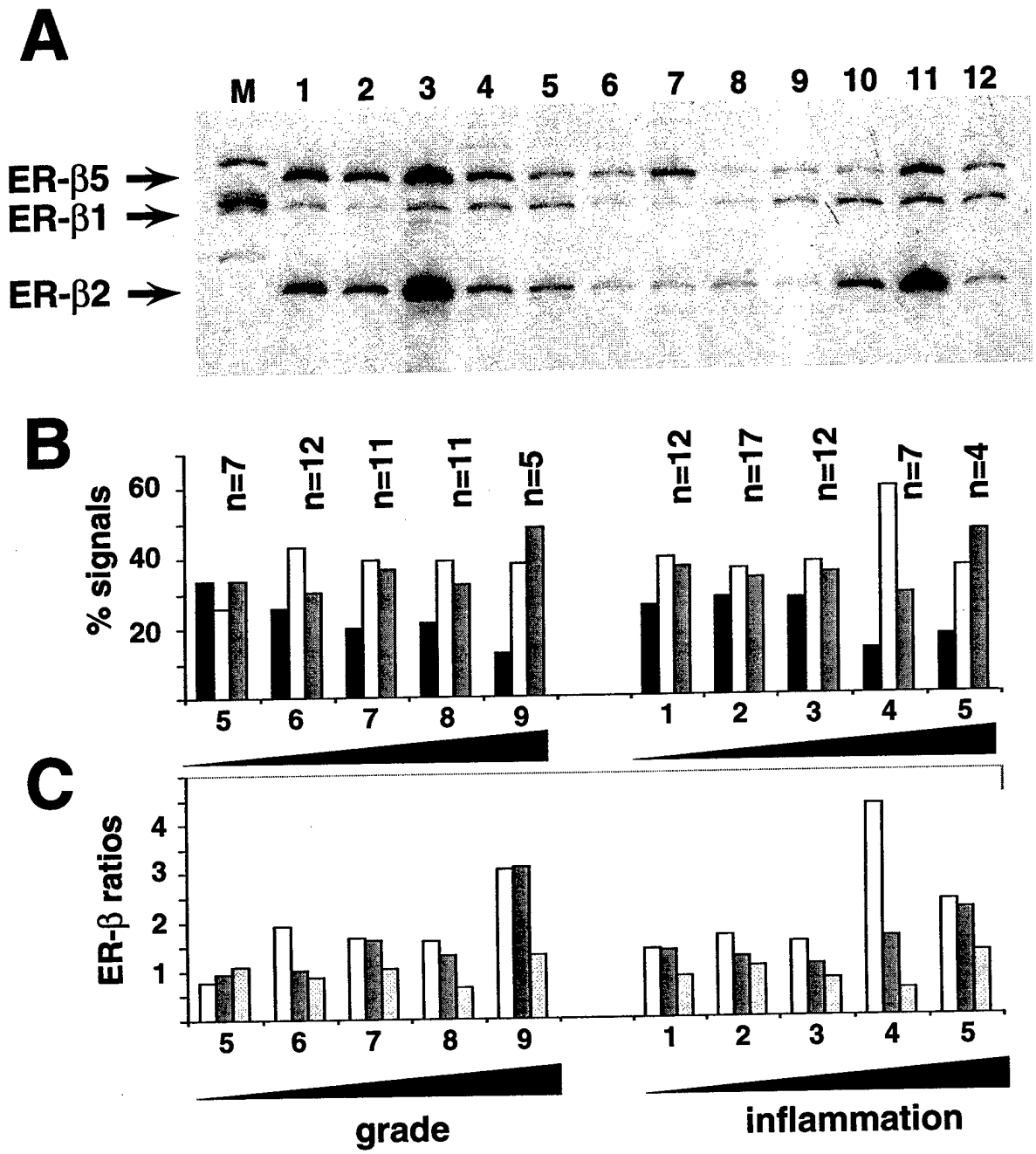
TP-PCR analysis of the relative expressions of ER–β1, ER–β2 and ER–β5 mRNAs within a cohort of 53 independent breast tumors. Total RNA was extracted from 53 breast tumors, reverse transcribed and analyzed by TP-PCR as described in the Materials and Methods section. PCR products were separated on acrylamide gels. **A:** Autoradiogram showing the results obtained for 12 cases (1-12). M: molecular weight marker ϕx174 RF DNA/Hae III fragments. **B:** ER–β1, ER–β2 and ER–β5 signals have been quantified and expressed relatively to the sum of the signals obtained, as described in the Materials and Methods section. Tumors have been sorted according to their Notthingham grade scores (5-9) or to their levels of inflammation (1-5). For each group, the number of patients (n) and the medians of the relative expression of ER–β1, ER–β2 and ER–β5 signals are indicated by black, white and dark gray bars, respectively. **C:** For each group, the number of patients (n) and the median of the ratios ER–β2/ER–β1, ER–β5/ER–β1 and ER–β5/ ER–β2 signals are indicated by white, dark gray and light gray bars, respectively.

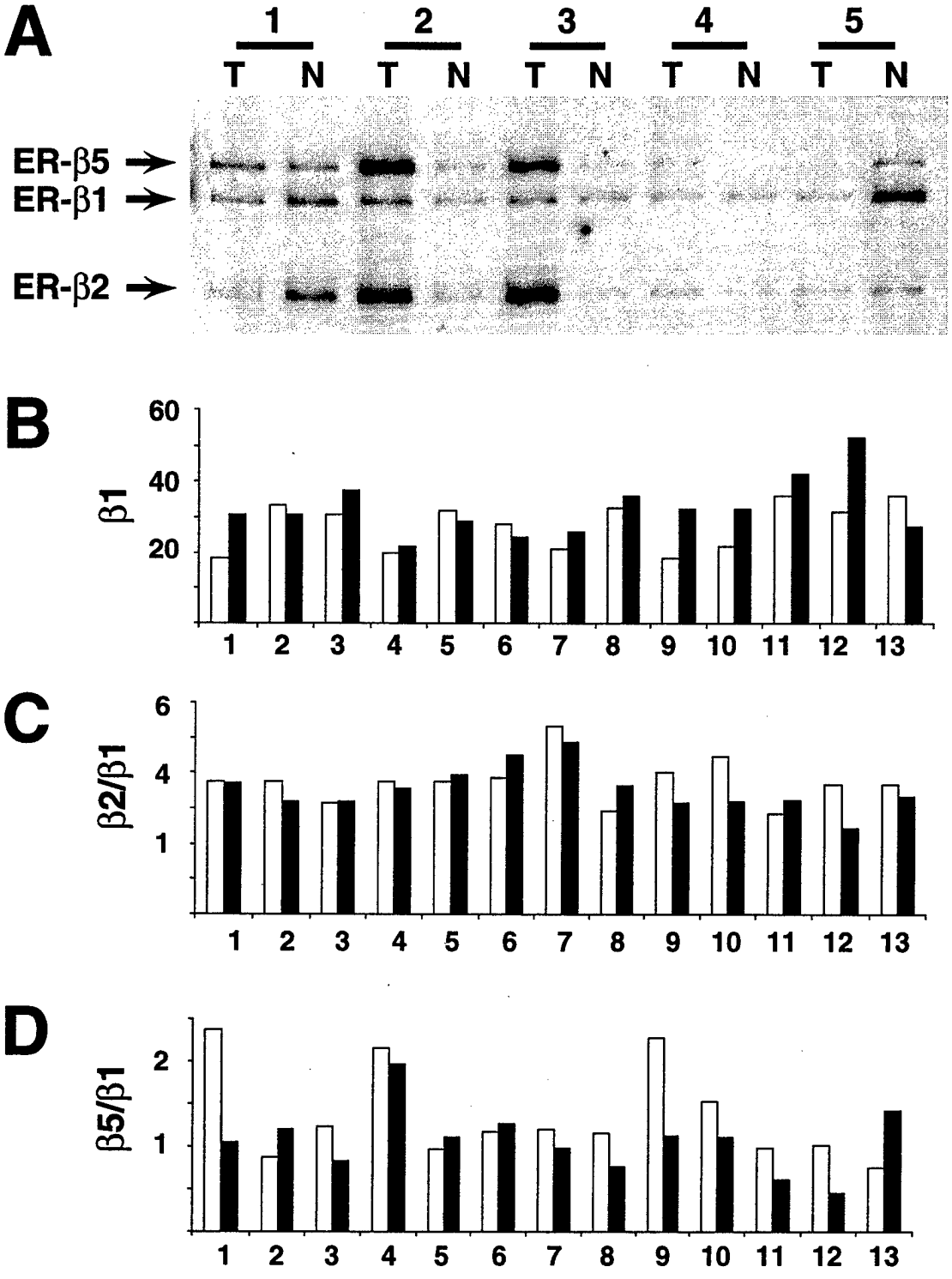
Fig. 4

TP-PCR analysis of the relative expressions of ER–β1, ER–β2 and ER–β5 mRNAs within matched normal and tumor compartments of human breast tumors. Total RNA was extracted from 13 breast tumors (T) and adjacent normal breast tissues (N), reverse transcribed and analyzed by TP-PCR as described in the Materials and Methods section. PCR products were separated on acrylamide gels. **A:** Autoradiogram showing the results obtained for 5 cases (1-5). **B:** ER–β1, ER–β2 and ER–β5 signals have been quantified and expressed relatively to the sum of the signals obtained, as described in the Materials and Methods section. For each case (1-13) the relative percentages of ER–β1 in tumor (white bars) and normal (black bars) components are shown. **C:** For each case (1-13), the ratios ER–β2/ER–β1 in tumor (white bars) and normal (black bars) components are shown. **D:** For each case (1-13), the ratios ER–β5/ER–β1 in tumor (white bars) and normal (black bars) components are shown.


A
ER-β1 protein
A/B
AF-1
C
DBD
D
E
LBD
F
mRNA
1
2
3
4
5
6
7
8
β1U
β1L
ER-β1
7
8
268 bp
β2L
ER-β2
7
214 bp
ER-β5
7
295 bp
ER-β4
7
529 bp
B
M
MDA MB 231
MDA MB 468
ZR75
BT 20
T-47D
MCF 7
529 bp
ER-β5
295 bp
ER-β1
268 bp
ER-β2
214 bp


A
β5 1 1 1 1 1 1 1 0 0
β1 1 1 1 1 1 1 0 1 0
β2 0 0.2 0.4 1 4 8 0 0 1
ER-β5
ER-β1
ER-β2
B
%signal
r = 0.996
p < 0.0001
β5%input
r = 0.995
p < 0.0001
β1%input
r = 0.998
p < 0.0001
β2%input


A
M 1 2 3 4 5 6 7 8 9 10 11 12
ER-β5
ER-β1
ER-β2
B
% signals
60
40
20
n=7
n=12
n=11
n=11
n=5
n=12
n=17
n=12
n=7
n=4
5 6 7 8 9
1 2 3 4 5
C
ER-β ratios
4
3
2
1
5 6 7 8 9
1 2 3 4 5
grade
inflammation


A
1
2
3
4
5
T N T N T N T N T N
ER-β5
ER-β1
ER-β2
B
β1
60
40
20
1 2 3 4 5 6 7 8 9 10 11 12 13
C
β2/β1
6
4
1
1 2 3 4 5 6 7 8 9 10 11 12 13
D
β5/β1
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13